GERMLINE MUTATIONS AND REPRODUCTIVE EFFECTS IN FATHEAD MINNOWS EXPOSED TO CONTAMINATED SEDIMENTS FROM RANDLE REEF, HAMILTON HARBOUR

By

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Abstract

Sediments at Randle Reef, Hamilton Harbour are highly contaminated with coal tar, a complex mixture of polycyclic aromatic hydrocarbons (PAH's). Studies there have demonstrated adverse effects in fish, including elevated mortalities and increased incidences of tumors. However, reproductive effects and heritable genetic alterations have not been evaluated. We exposed sub-adult fathead minnow (*Pimephales promelas*) to contaminated water and sediments at Randle Reef, Hamilton Harbour using two approaches. In the first, fish were caged at Randle Reef for six weeks, and in the second fish were exposed in-lab to whole sediments for three weeks. Fish from both studies were transferred to clean aquaria and evaluated for reproductive impairment immediately following exposures. We also determined germline mutation rates in fish exposed in-lab, using microsatellite DNA markers. Overall, we found no evidence to support our initial expectation that fish exposed to the highly contaminated sediments at Randle Reef would experience elevated germline mutations and reproductive impairment. This finding was unexpected, given the large number of studies that have reported effects following exposure to PAH's and PAH-contaminated sediments, including sediments contaminated with coal tar. A number of factors that may have contributed to results obtained are discussed.

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I'm writing this one day after completing the thesis final draft and a short time away from the defence. It's been a very long road (32 months!). I had my doubts along the way whether this project would ever end, or if I would ever make it to the finish line.

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Chapter 1: Introduction

Many laboratory studies and field surveys have been carried out in recent decades to determine whether and to what extent fish and wildlife are being adversely impacted by environmental contamination. This is no surprise, given the vast number of contaminated sites in North America alone. In the United States, 1255 sites are currently designated as "Superfund Sites" due to substantial site contamination and potential risks to humans and wildlife (USEPA, 2009). An additional 43 sites located within the Great Lakes Basin have been designated as Areas of Concern (AOC) by the International Joint Commission due to impairment of 14 key beneficial uses (IJC, 2003). In Canada, over 19,000 sites have been classified as contaminated or are suspected to be contaminated, the classification being limited to only include federally-owned land or land for which the federal government has committed to provide funds for remediation efforts (TBC, 2009).

Hamilton Harbour (Lake Ontario) was designated an AOC in 1985, due to impairment in 11 of the 14 key beneficial-use criteria used by the IJC (1999). Some of those beneficial uses are indicators of serious degradation of ecosystem health that may also have implications for human health. For example, beneficial use impairment in Hamilton Harbour includes fish tumors and deformities, bird or animal deformities or reproductive problems, degraded fish and wildlife populations, and restrictions on fish and wildlife consumption.

Numerous studies have investigated the health and reproductive effects in wild or sentinel fish and animals exposed to contaminants in Hamilton Harbour. For example, brown bullhead (*Ameiurus nebulosus*) collected from Hamilton Harbour exhibited elevated skin and liver neoplasms as well as elevated DNA damage within erythrocytes, compared with fish from reference locations (Hayes et al., 1990; Pandrangi et al., 1995). Krantzberg and Boyd (1992) measured elevated body burdens of polycyclic aromatic hydrocarbons (PAH's) in fathead minnows (*Pimephales promelas*) and mayfly nymphs (*Hexagenia limbata*) exposed to Hamilton Harbour sediments collected from various locations, and mortality rates were elevated in individuals exposed to sediment from

several of these sites. Kavanagh et al. (2004) found increased proportions of inter-sex male white perch (*Morone americana*) in the adjacent Cootes Paradise relative to reference fish, but could not make strong conclusions with regards to causative agents. Carcinogenicity and mutagenicity of extracts of sediment from Hamilton Harbour has been documented (Metcalfe et al., 1990; Balch et al., 1995), and extracts from zebra mussels (*Dreissena polymorpha*) collected from the harbour have also shown mutagenic potential (Marvin et al., 1994).

Heritable alterations and reproductive effects have also been observed. For example, herring gulls (*Larus argentatus*) nesting in the vicinity of the harbour exhibited elevated germline DNA mutations relative to gulls from rural locations (Yauk and Quinn, 1996; Yauk et al., 2000). Snapping turtle (*Chelydra serpentine*) eggs collected from the Hamilton Harbour basin contained elevated burdens of organochlorine pesticides and polychlorinated biphenyls (PCB's), and hatchlings exhibited altered sexually dimorphic morphology and decreased hatching success compared to eggs and hatchlings from reference sites (de Solla et al., 2002, 2007, 2008).

One area of Hamilton Harbour that has received a lot of attention from both the media and the scientific community is the area commonly known as Randle Reef. Sediment contamination at Randle Reef is severe, exceeding contamination levels at several other Areas of Concern (Murphy et al., 1990) and acute toxicity in both fish and invertebrates has been demonstrated (Murphy et al., 1990; McCarthy et al., 2004). Sediments at this location are primarily contaminated with coal tar (Marvin et al., 2000), a complex and variable mixture of high- and low- molecular weight polycyclic aromatic hydrocarbons (PAH's). Concentrations of some metals such as Zn, Pb, Ni, Cu, and Cr are also high relative to reference sites, and a few of these are present at levels which exceed sediment quality guidelines (Murphy et al., 1990; Krantzberg and Boyd, 1992; Borgmann and Santiago, 2004). PCB's are also present (Munkittrick et al., 1995), and various other contaminants are expected to be present due to runoff from roadways, combined sewer overflow outfalls, and municipal sewage treatment plant effluent discharges.

While several diverse classes of contaminants have been identified at Randle Reef, PAH's have been studied most frequently. This is likely due to the extreme concentrations of PAH's in Randle Reef sediment samples, relative to other sites in the harbour or elsewhere. The spatial extent of the contamination is also large, with an estimated 37,000 m^3 of sediment containing in excess of 200 μg total PAH's per gram of sediment, and total PAH's exceed 800 μg/g in some samples (Murphy et al., 1990). By comparison, bottom sediments from other regions of the harbour typically have PAH concentrations one or two orders of magnitude lower (Marvin et al., 2000).

Fish exposed to PAH-contaminated sediments or water have clearly demonstrated adverse effects, including elevated mortality (Roberts et al., 1989; Bickham et al., 1998), DNA damage or alteration (Bickham et al., 1998; French et al., 1996; Ericson et al., 1998; Hose and Brown, 1998; Aas et al., 2001; Kilemade et al., 2004), external and internal lesions, tumors, and neoplasms (Ericson et al., 1998; Vogelbein et al., 1990; Huggett et al., 1992; Baumann, 1998), and immune system dysfunctions (Huggett et al., 1992; Karrow et al., 1999). Reproductive effects have also been seen, including decreased fecundity (Nye et al., 2007), increased embryo and larval mortalities (Kocan et al., 1996; Nagler and Cyr 1997; Vines et al., 2000), and elevated birth defects and abnormalities (Vines et al., 2000; Wassenberg and Di Giulio, 2004).

Adverse effects have also been seen in fish exposed to contaminated Randle Reef sediments. For example, Leadly et al. (1999) exposed brown bullhead (*Ameiurus nebulosus*) to sediment collected at Randle Reef and found elevated mortality and elevated EROD activity after 72 hours of exposure. McCarthy et al. (2004) found 100% mortality in juvenile fathead minnows exposed to contaminated sediment collected from Randle Reef, whereas fish exposed to sediment collected in Lake Erie experienced no mortalities. However, the potential for reproductive disruption and heritable effects in fish from this area has not been investigated.

While many studies have looked at the impacts of Hamilton Harbour contaminants on specific health measures such as tumor prevalence, reproductive performance, or gross physiological abnormalities, few have investigated the long-term,

multigenerational effects that may be at play. Some studies have measured genotoxic endpoints such as mutagenicity using the Ames test, prevalence of DNA adducts, and incidence of DNA strand breaks in wild or sentinel species exposed to various contaminants in the harbour (see previous sections). Such findings may have implication for genetic health, but they say nothing as to whether the effects are transient, occurring only in the current generation, or whether they are heritable and can be passed to future generations.

Studies in Hamilton Harbour and elsewhere have been almost exclusively limited to adverse effects seen in the present generation. Most have evaluated sensitive endpoint while the organism is still being exposed to the contaminants, or shortly after exposure. Unfortunately, few studies have evaluated effects from a population perspective, and fewer still have looked for multigenerational effects. This is an important distinction. For example, the rate of particular health effects that occur only in exposed individuals and are not heritable or do not have direct effects on reproduction may decline following reduction in contaminant inputs or site remediation (Baumann and Harshbarger, 1995; Myers et al., 2008). While such a decline would be encouraging, that information would not reveal if effects at the population level have occurred, and by extension whether the effects of the contaminants have long-term carry over effects even after they have been removed from the ecosystem.

Various techniques have been used over the past decades to study heritable changes in DNA in fish. The utility of a class of genetic markers collectively referred to as tandem repeat DNA became apparent to fisheries managers and fish biologists during the late 1980's and early 1990's, first for use in population and genetic diversity studies (Wright and Bentzen, 1994) and later as a marker for mutation induction. These elements are unstable relative to most commonly used DNA markers, and that leads to a high degree of variability within and among populations. The instability of tandem repeat sequences translates into higher mutation rates compared to other markers, and this in turn reduces the number of individuals required to obtain statistically significant results in mutational studies (Yauk, 2004).

Three major classes of tandem repeat DNA have been identified, although there is no full consensus on definitions for each class (Chambers and MacAvoy, 2000). The smallest are the microsatellites, tandem repeats of core sequences between 2 and 6 bps, with sizes typically in the range of 100-600 bps. Minisatellites are similar to microsatellites but have longer core repeats between 10 and 100 bps and a greater number of repeats, with sequence lengths of several kbs (Yauk, 2004; Chambers and MacAvoy, 2000). Expanded Simple Tandem Repeats (ESTR's) are similar to minisatelites in terms of length but have core repeat sequences of less than 10 bps which is more in line with microsatellites (Yauk, 2004). To date, only microsatellites and minisatellites have been identified in fish.

Historically, minisatellites and ESTR's have been more often used to measure mutations rates at tandem repeat DNA loci following environmental or experimental exposure to radiation or toxic chemicals. However, microsatellites are increasingly being used due to several key advantages. First, microsatellites have become the marker of choice in fish population studies (Wright and Bentzen, 1994; O'Connell and Wright, 1997; Neff et al., 2000), which in turn has resulted in the availability of more sequence information and the publication of background mutation rates for several fish species. Second, microsatellites are amplified using the polymerase chain reaction, followed by separation on high percentage polyacrylamide gels. This allows for the detection of even the smallest changes in allele size, such as the loss or gain of as few as one or two base pairs. Thirdly, microsatellites are more reliable when comparing between gels, since a more accurate determination of allele sizes can be made for microsatellites as compared to the longer minisatellites (O'Connell and Wright, 1997; Somers, 2006). Finally, microsatellites lend themselves to automated separation and detection techniques. This is in contrast to minisatellites and ESTR's which require manual separation on agarose gels followed by southern blotting and probing.

A few studies in the Hamilton Harbour region have examined DNA damage from a heritability perspective using two different types of tandem repeats: minisatellite DNA and ESTR DNA. In the initial studies, herring gulls (*Larus argentatus*) nesting within the

harbour region were found to have elevated germline mutations in minisatellite DNA compared with gulls nesting in rural sites (Yauk and Quinn, 1996; Yauk et al., 2000). Follow-up studies with mice showed experimentally that airborne particulate matter was at least in part responsible for ESTR germline mutations, but did not completely rule out diet as a source of genotoxins contributing to germline mutations (Somers et al., 2002, 2004, 2008).

Investigations into the health impacts or potential for health impacts at contaminated sites have used three predominant strategies for measuring biological endpoints in exposed animals. The first involves the sampling of wild animals captured at the site(s) of interest. While that may give a more realistic picture of effects in populations inhabiting the contaminated sites, a high degree of variability is often encountered due to variations in age, catch effort, and also due to movement patterns such as seasonal migration (Crane et al., 2007). The second method, *in-situ* testing, involves caging lab animals on-site for a defined period followed by sampling or further in-lab testing. That strategy allows for a certain degree of standardization among individuals, but environmental variability may still be problematic (Burton, 1991; Burton et al., 2005). Additionally, that method is often logistically challenging and may be too stressful or logistically impossible for some species (Crane et al., 2007). The third and most widely employed method in aquatic toxicology is to collect contaminated media (water, sediment, etc.) from the study sites, and to expose either wild-caught or, more commonly, laboratory reared organisms in a lab setting. While it may provide for a less realistic exposure as compared to the other two methods, it is often more feasible, and removes a great deal of variability that cannot be controlled in the field (Burton et al., 2005). As such, results are often more reproducible and more informative, although extrapolation of effects from the lab to the field may be invalid due to environmentally unrealistic exposure conditions (Crane et al., 2007).

Fathead minnows (*Pimephales promelas*) are frequently used as a sentinel species when testing for biological effects resulting from exposure to environmental contaminants, for several reasons. First, they are ubiquitous across a large part of North

America. Fathead minnows and their related *Cyprinid* counterparts account for a large percent of the species and individuals present in many aquatic ecosystems and occupy an important position if many aquatic food chains (Ankley and Villeneuve, 2006). Importantly, though, they are a commonly used fish in aquatic toxicology and in sediment testing (Burton, 1991; Ankley and Villeneuve, 2006; Teather and Parrott, 2006), and a great wealth of reproductive, physiological, and toxicological information exists (USEPA, 1996; Jensen et al., 2001; Watanabe et al., 2007). They are considered hardy against handling stress and some adverse conditions such as low dissolved oxygen, low pH, and elevated ammonia (Mount, 1973; Croke, 2001; Kahl et al., 2001). Finally, they are easily cultured in the lab, and mature fish readily breed with only minimal requirements.

Fathead minnows are fractional asynchronous breeders, meaning they will produce several clutches of offspring over the duration of the breeding season, with variability in onset and duration of the breeding cycle occurring among individuals (Gale and Buynak, 1982). In natural settings, male fathead minnows will select and guard a breeding substrate such as a submerged log. The male will then court a female, and if successful the female will lay eggs on the underside of the substrate, which is quickly followed by fertilization of the eggs by the male (Cole and Smith, 1987). The female then leaves, and the male guards the eggs until they hatch (Divino and Tonn, 2008). Males and females will mate with multiple partners throughout the breeding season, and alternative reproductive tactics such as sneak fertilization have been described (Bessert et al., 2007).

Objective

Fish inhabiting the contaminated Randle Reef area of Hamilton Harbour are probably being exposed to high concentrations of PAH's and to a lesser extent other environmental contaminants. PAH's have proven to be genotoxic and mutagenic in fish and other organisms. Given that mice and gulls exposed to contaminants near Hamilton Harbour showed elevated germline mutations, I predicted that fish exposed to the contaminated sediments at Randle Reef would also experience elevated germline mutations as well as other reproductive and health effects. To that end, I exposed fish to

the contaminated sediments near Randle Reef, evaluated reproductive performance in the absence of contamination, and finally measured and compared germline mutation rates between the exposed and non-exposed groups. For this study, only the "*in-situ*" and "in-lab" methods were suitable for breeding of exposed adults under clean conditions. Given the advantages and limitations of these two methods, I decided to conduct two exposure experiments, one in which the fish were exposed *in-situ*, and the other in which the fish were exposed in-lab. This duplication of effort was intended to add robustness to the study design, increasing the ability to distinguish artifacts from real and environmentally relevant effects.

My null hypothesis was that fish exposed to Randle Reef sediment and water-borne contaminants will be similar to reference or control groups in terms of general health, reproductive performance, and germline mutations rates. My expectation, based on available evidence, was that the types and magnitude of contamination at Randle Reef were sufficient to affect the general health of exposed fish, and to cause reproductive impairment such as decreased egg production, egg hatch, or increased larval mortality or abnormalities, and finally, that offspring born to Randle-exposed fish would show higher levels of inherited DNA mutations relative to reference or control groups.

Chapter 2: *In-situ* exposure of fish to contaminated sediments at Randle Reef, Hamilton Harbour

Methods

Fish

Two hundred and fifty 2.5-month old juvenile fathead minnows (*Pimephales promelas*) were obtained from a commercial supplier (Aquatic BioSystems Inc, Fort Collins, CO, USA) and maintained in a 200-L fiberglass tank for six weeks. Dechlorinated tap water was delivered to the tank at 150-200 ml/min (1-2 water changes per day); the water was kept at 23 ± 1°C and water chemistry of influent water was determined monthly throughout the duration of the experiment (Table 2.1). The fish were provided frozen brine shrimp (FBS; San Francisco Bay Brand, Newark, CA, USA) and allowed to feed to satiety twice daily.

Caging and site details

Galvanized wire mesh (1/8") minnow traps (MT18; Dynamic Aqua-Supply Ltd, Surry, BC, Canada) were modified by folding the opened ends closed and sealing any gaps or sharp edges with silicon caulking. A custom-built stainless steel mooring held five replicate cages upright in a circular fashion (Figure 2.1). The mooring was designed to hold the cages at a constant height of 1 meter above the sediment to allow for sufficient exposure to the contaminants in the sediment while reducing the risk of fish mortality due to sediment upwelling.

One mooring of five replicate cages was used at Randle Reef (UTM coordinates 17T 594716 4791830) and another at a field control site near LaSalle Marina, Hamilton Harbour (UTM coordinates 17T 594131 4 794969) (Figure 2.2). Both sites were 8.5 ± 0.5 meters deep.

Fish were weighed and randomly divided into 15-L aquaria (15 fish each) two weeks prior to exposures. Water temperatures were lowered 0.5°C per day until similar to

prevailing field conditions (20°C). Each aquarium received water at 30-45 ml/min and the feeding rate was set at 0.5 ml FBS per tank, twice daily. Tanks were randomly assigned to treatments, with five replicate tanks for each of the three treatments. Fish were not weighed again prior to exposures to minimize handling stress.

Fish were transported to field sites in food-grade polyethylene bags filled with dechlorinated tap water. Field sites were close to the lab site (Figure 2.2) and transport from the lab to the field sites took less than 60 minutes. Fish within tanks assigned to control treatment replicates were not disturbed except to provide food.

Fish were maintained in the cages for six weeks (13-Sept to 25-Oct, 2007). Sites were visited 2-3 times per week to monitor fish health, provide food, and measure water quality parameters. On each visit, cages were raised to the surface and dead, visibly ill, or injured fish were removed. Frozen brine shrimp wrapped in fine nylon mesh (2 x 5 ml) was suspended inside cages on each visit. The feeding rate was increased over the exposure period to a final rate of 16 ml per cage per visit. Water temperature, dissolved oxygen, pH, and conductivity were measured at cage depth (7.5 meters) on each visit (YSI 6600 Sonde and 650MSD handheld meter; YSI Inc., Yellow Springs, OH, USA).

Fish within lab control replicates (5 replicates, 15 fish per replicate) were maintained in 15-L glass aquariums throughout the field exposure period. They were fed as described for the field exposed fish, but at half the rate (5 ml per tank per visit, increasing to 8 ml per tank per visit) because I assumed that half of the brine shrimp provided to field groups would be washed out of the exposure cages. Control tanks were continuously aerated, supplied with dechlorinated tap water (30-45 ml/min), and temperature and photoperiod were set to mimic field conditions. Activity near the laboratory control tanks was minimized and the control fish were only disturbed on days when the field fish were visited.

Post-exposure measures and breeding

Following the 6-week exposure period, fish were transported back to the lab in food-grade polyethylene bags filled with site water. Handling and transport time was less than 60 minutes. Once in the lab, fish were transferred into15-L glass aquariums that

were similar to the lab control tanks in terms of water temperature, air delivery, and water supply. All fish were weighed and 3 fish per replicate were randomly selected and dissected on the following day. Body weight (BW, grams), gonad weight (GW, grams), liver weight (LW, grams), and standard length (SL; tip to tail, cm) were determined for each fish. Gonadal Somatic Index (GSI; GWx100/BW), Liver Somatic Index (LSI, LWx100/BW), and Condition Factor (K, BWx100/SL^3) were calculated. Secondary sex characteristics (fatpads, nuptial tubercles, and dorsal banding patterns in males; extended abdomens and elongated ovipositors in females) were recorded as present or absent.

The remaining 11 or 12 fish per tank were maintained for 3 months to assess reproductive performance and offspring viability. Water temperature was raised 0.5°C per day until the average temperature was 24°C. Fish were provided brine shrimp twice daily, at a rate of 2.0 ml per tank per feeding during the first four weeks, and 3.0 ml per tank per day thereafter. Dechlorinated water was delivered at a rate of 35-50 ml/min with continuous aeration.

Three breeding tiles (half-sections of PVC drain pipe) were placed in each tank on day 6 post-exposure. Because fathead minnows are fractional spawners, and reproductive pairs typically produce an average of 85 and up to 480 eggs every 3 to 4 days (Jensen et al., 2001; Gale and Buynak, 1982), tiles were checked every 3 or 4 days until onset of breeding, after which they were checked daily. Tiles containing eggs were removed and immediately replaced with clean tiles. Eggs were carefully rolled from tiles, counted and assessed for fertility and viability, and a subset of viable eggs, to a maximum of 100 eggs was reared in eggs cups within separate tanks at 25°C. The fry were assessed following yolk-sac absorption at 7-8 days post-fertilization. Normal live fry, dead fry, dead eggs, and abnormal fry were counted, and common physical abnormalities (yolk-sac and pericardial edema, spinal and craniofacial deformities, microphthalmia, anophthalmia) were recorded. Fry were preserved in absolute ethanol and stored at room temperature.

Reproductive assessment was carried out for three months following exposure, after which time all adult fish were weighed but not dissected. Instead, adults were divided into breeding pairs and allowed to breed for an additional 40 days. The sole

purpose of this "pair-breeding" period was to provide additional samples for future DNA analysis; reproductive output data was collected but not analyzed. All adults were euthanized and dissected immediately following the pair-breeding period, as previously described. White muscle, liver, and eye tissues were preserved in absolute ethanol and stored at room temperature for future DNA analysis.

Statistical Analysis

Nested analysis of variance was used to compare reproductive and physiological endpoints among treatments, with replicates (tanks or cages) nested within treatments. In the Nested ANOVA, variation among replicates was first tested ($F_{replicate} = MS_{replicates} / MS_{error}$). When no significant variation was found within replicates ($F_{replicates} < F_{critical}$), the F-statistic was calculated as $F_{treatment} = MS_{treatment} / MS_{error}$. When a significant variation was found among replicates, the F-statistic was adjusted to account for this variation ($F_{treatment} = MS_{treatment} / MS_{replicates}$).

All data were tested to ensure that the assumptions of the ANOVA were met (homogeneity of variance, residuals normally distributed, skewness and kurtosis within acceptable range) for each treatment group. When necessary, data were transformed using a logarithmic (base 10) or square-root transformation. Data that still failed to meet the assumptions even after trials with additional transformations were tested using the Kruskal-Wallis non-parametric analysis of variance. In such cases, each treatment group was first tested with the Kruskal-Wallis test to ensure that replicates within treatments were similar. In all such cases no significant differences were found, and all values were then pooled between replicates within treatments.

When a significant difference was found among treatments, means were compared using Tukey's all-pairwise comparisons (ANOVA) or the Kruskal-Wallis all-pairwise testing procedure provided in the Statistix software.

All statistical calculations were performed using Statistix 9 (Analytical Software, Tallahassee, FL, USA), except for data exploration (testing for the assumptions of ANOVA) and contingency table testing which were performed in Systat 11 (Systat

Software Inc, Chicago, IL, USA). For all tests, α was set at 0.05. All data are presented as the mean ± SEM (standard error of the mean) unless otherwise indicated

Results

Site characteristics

Initial temperature at each site was 19-20°C and declined to around 16°C by the end of the exposure period (Figure 2.3A). While both field sites were usually within 0.5°C of each other on any given visit, lab temperatures tended to be 1-2°C higher (Figure 2.3A). Daily temperature fluctuation was not recorded in this study, but 24-hour temperature fluctuations recorded hourly at cage depth during a preliminary study from 5-Oct to 10-Nov 2006 were typically less than 0.5°C at both sites (Miller, unpublished data).

Dissolved oxygen was consistently above 6.0 mg/L at both sites except for two low readings of 4.4 and 5.5 mg/L at Randle Reef (Figure 2.3B). Dissolved oxygen in lab control tanks was always high due to continuous aeration and water exchange (data not shown). Field site pH varied between 8.0 and 9.0 and was only slightly higher at LaSalle (Figure 2.3C), whereas lab control tanks tended to have pHs between 7.0 and 8.0 (data not shown). Specific conductivity fluctuated between 480 and 550 μS/cm but did not differ greatly between sites on any visit (Figure 2.3D)

Adults

Initial total fish weight per replicate was 5.81 g (±0.17 SEM; 15 fish per replicate) and did differ among treatments (ANOVA, $F = 0.67$, $d.f. = 2,12$; $p = 0.531$). Fish were not weighed again prior to deployment to minimize handling stress.

There was complete survival in all replicates except for two LaSalle cages and one Randle cage which each had single mortalities. Single fish were missing from two other Randle cages and likely escaped during the exposure, as no carcasses were found in these cages on any visit. Single fish were missing from two control aquaria; one fish was

found and euthanized after it had jumped from one of the control tanks during the exposure period.

Average fish weight increased in all treatments, with significantly greater weight gains seen in Randle and LaSalle replicates compared to control replicates (Figure 2.4). Average weight following exposure was 1.8 - 1.9 times higher in LaSalle and Randle replicates compared to the control replicates (Figure 2.4).

Fish were still immature after the six-week exposure period, and it was not possible to distinguish males from females by using external morphology. Typical male and female secondary sex characteristics (fatpads, nuptial tubercles, and dorsal banding patterns in males, or extended abdomens and elongated ovipositors in females) were absent in all individuals.

Subsets of three randomly selected fish per replicate were dissected immediately following the exposure. Of the 45 fish dissected, 29 (64%) were classified as female (based on gonadal morphology), while only 8 (18%) were classified as male. The 8 remaining fish (18%) could not be classified as male or female due to underdeveloped gonads, and were excluded from the analysis. That may have resulted in a systematic bias if, for example, small males tended to be excluded. However, the distribution of males, female, and unclassified fish among treatments was not significantly different ($\chi^2 = 3.69$, $d.f. = 2$; $p = 0.073$; Figure 2.5).

Female body weight and GSI differed significantly among treatments (Nested ANOVA, $F = 10.58$, $d.f. = 2,16$; $p = 0.001$, and $F = 5.03$, $d.f. = 2,14$; $p = 0.023$ respectively). LaSalle and Randle females had similar body weights (Tukey's, $p > 0.05$) and both groups were significantly larger than control females (Tukey's, $p < 0.05$; Figure 2.6A). GSI scores were significantly higher in LaSalle females compared to control females (Tukey's, $p < 0.05$) but did not differ between Randle and control females (Tukey's, $p > 0.05$) or between Randle and LaSalle females (Tukey's, $p > 0.05$) (Figure 2.6B). Female LSI and body condition (K) did not differ among treatments (Nested ANOVA, $F = 0.06$, $d.f. = 2,15$; $p = 0.937$, and $F = 2.64$, $d.f. = 2,16$; $p = 0.132$ respectively; Figure 2.6). Males did not differ among treatments for body weight, GSI,

LSI, or K (ANOVA, $p = 0.916$, $p = 0.352$, $p = 0.769$, and $p = 0.163$ respectively; Figure 2.6).

Post-exposure breeding

The number of male and female fish varied among tanks, both at the initiation of breeding due to random assignment of fish to treatments without knowledge of gender, and throughout the breeding phase due to mortalities. However, the proportion of fish that died in each treatment was similar among treatments ($\chi^2 = 0.61$, $d.f. = 2$; $p = 0.737$) and the time to death of fish did not vary by treatment (Logrank Test, $\chi^2 = 0.48$, $d.f. = 2$; $p = 0.788$; Figure 2.7). Similar results were obtained when males and females were separately tested (not shown). More importantly, the number of female reproductive days (the sum of the total number of days each female was present within a given tank. For example, if a tank had 6 females on day 1 post-exposure, and all females survived to the termination of the breeding trial on day 90-PE except one female that died on day 10 post-exposure and a second that died on day 22 post-exposure, the number of female reproductive days for that tank would be 4x90+10+22 = 392) did not differ significantly among exposures (ANOVA, $F = 0.49$, $d.f. = 2,12$; $p = 0.627$; Figure 2.7)

Total egg production differed significantly among treatments (ANOVA, $F = 4.81$, $d.f. = 2,12$; $p = 0.029$; Figure 2.8); production by Randle and LaSalle groups was similar (Tukey's, $p > 0.05$), and both produced significantly more eggs than the control groups (Tukey's, $p < 0.05$). However, two of the five control replicate tanks produced no eggs during the 3-month breeding period, and total egg production per replicate tank was similar among treatments when these two groups were excluded from the analysis (ANOVA, $F = 2.17$, $d.f. = 2,10$; $p = 0.164$).

Fecundity (eggs per female reproductive day) also differed significantly when all replicates were used (ANOVA, $F = 5.91$, $d.f. = 2,16$; $p = 0.016$; Figure 2.8), but was similar among treatments following exclusion of the two non-producing control tanks (ANOVA, $F = 2.77$, $d.f. = 2,10$; $p = 0.110$).

Average egg fertility and viability was high in all replicates (Figure 2.9) and did not vary among treatments (ANOVA, $F = 2.05$, $d.f. = 2,10$; $p = 0.180$, and $F = 0.52$, $d.f. = 2,10$; $p = 0.612$ respectively).

Egg hatching success was also high and did not vary among treatments (Kruskal-Wallis, $F = 1.46$, $d.f. = 2,10$; $p = 0.277$; Figure 2.9). Few dead fry were present at enumeration (24-48 hours post-hatch) and no difference in fry mortality was observed between treatments (Kruskal-Wallis, $F = 1.06$, $d.f. = 2,10$; $p = 0.335$; Figure 2.9). Roughly 1% of all fry were abnormal, with similar rates among treatments (Kruskal-Wallis, $F = 0.48$, $d.f. = 2,10$; $p = 0.630$, Figure 2.9).

Adults were dissected immediately following the 40-day pair-breeding phase. Only adults from successful breeding pairs were included in the analysis, to minimize variance in endpoints such as GSI and LSI that might be altered due to lack of reproductive activity. All endpoints tested (weight, GSI, LSI, K) were not significantly different among treatments in both males and females (Nested ANOVA, $p > 0.05$; Table 2.2).

Chapter 3: In-lab exposure of fish to contaminated sediments from Randle Reef, Hamilton Harbour

Methods

Sediment

Sediment was collected in December 2007 from seven sites near Randle Reef, Hamilton Harbour, and at seven sites near LaSalle Marina (Table 3.1). Collection points at Randle Reef were selected to be within the zone of highest PAH sediment contamination (Borgmann and Santiago, 2004). Collection points were chosen near LaSalle Marina to mimic the Randle sites with respect to depth and substrate type.

Approximately 8 litres of sediment were collected at each site using a Wildco Petite Ponar grab sampler (Wildlife Supply Company, Buffalo, NY, USA). Sediment was placed in plastic pails lined with food-grade polyethylene bags and stored at 4°C until mixing. Sediment collection at Randle Reef and LaSalle Marina occurred on consecutive days and all tools were thoroughly cleaned between days. Air temperatures were 1-2°C and skies were quite overcast on both days, thereby limiting thermolytic or photolytic reactions of the sediment samples.

Sediments from both locations were "organic mud", having a silt-clay type texture. Few large items (rocks, debris, bivalve shells) were found at either site, with the exception of zebra mussel (*Dreissena polymorpha*) shells which ranged from absent to common between sites in both locations. A small number of benthic invertebrates were noted, typically tubifex worms (both locations). Sediments from two of the Randle sites exuded a strong grease-like odour.

To prepare composite sediments, sediments from each pail were first passed through a sieve (7-mm square mesh aperture) to remove larger debris, combined, and thoroughly mixed. Since oxidation of the outermost layer of sediment in each pail was evident, based on coloration, I first removed and disposed of the uppermost 2-3 cm of sediment, and also excluded about 1 cm of sediment along the sides and bottom of the

pail. Once mixed, subsamples were preserved in pre-cleaned glass jars and stored at -20°C until chemical analysis. Stainless steel tools were used for all mixing and handling of sediment, and LaSalle sediments were mixed first to prevent contamination from Randle sediments.

Composite sediments were allocated to exposure tanks as follows: first, 1 litre portions of sediment were added to pre-weighed and numbered glass beakers. Beakers were re-weighed, covered with parafilm, and stored at 4°C for less than 24 hours prior to use.

Experimental Setup

Twelve 50-L tanks were arranged within a large water bath and supplied with dechlorinated tap water and air. Black plastic bags were used to line the exterior bottom and sides of each aquarium to reduce the disturbance to fish. It was also done because tanks without sediment would allow more light to pass into the tanks, particularly through the bottom glass, compared to tanks with sediment.

Each tank was randomly assigned to one of three treatments: Randle sediment (PAH-contaminated site; "Randle"), LaSalle sediment (harbour reference site; "LaSalle"), or no sediment (water control; "control"), giving four replicates per treatment. The contents of three sediment-filled beakers (each containing 1 litre of sediment; above) were added to each replicate tank, giving a total sediment volume of three litres per replicate. The average wet-weight of sediment added to tank each was 3,885 g (± 8.6 SEM) for LaSalle tanks, and 4,015 g (± 0.5 SEM) for Randle tanks. Dechlorinated tap water was used to rinse each beaker and the rinsate was also added to the corresponding aquarium. Thirty-six litres of dechlorinated tap water (~16°C) was carefully added (39-L control tank) and tanks were allowed to sit 48 hours in the dark at room temperature, after which water and air flow were initiated, and the tanks were left for an additional 96 hours. Water temperatures remained near room temperature during that period, and were increased to 23°C one day before initiation of exposures.

Fish

Two hundred and fifty 3.5 month old sub-adult fathead minnows (*Pimephales promelas*) were obtained from a commercial supplier (Aquatic BioSystems Inc, Fort Collins, CO, USA) and were held together in a 200-L fiberglass tank for one month prior to use. Water temperature was maintained near 23°C and dechlorinated tap water was provided at a rate sufficient to give approximately three complete water changes per day; water chemistry of influent water was determined monthly throughout the duration of the experiment (Table 2.1). The fish were provided frozen brine shrimp (FBS; San Francisco Bay Brand, Newark, CA, USA) and allowed to feed to satiety twice daily.

Exposure

Fish were individually weighed and divided among the 12 replicate tanks using a predetermined randomized order. Twenty-two fish were assigned to each tank. Tanks were maintained near 24°C for the duration of the exposure. Flow rates were initially low (40-60 ml/min) but were increased to 80-100 ml/min shortly after the fish were introduced to maintain acceptable water quality. The initial photoperiod was 14:10 (hours light:dark) and was adjusted to 15:9 after one week and to 16:8 after two weeks. Partially melted FBS was provided to each tank twice daily at a rate of 4.5 ml FBS per feeding, which was increased by 0.5 ml per feeding in the second and third week. Temperature and water supply flow rates were checked daily and free ammonia was measured twice weekly (MultiTest Ammonia; Seachem Laboratories Inc, Madison, GA, USA). Dissolved oxygen, pH, and conductivity were measured on exposure-day 10 and 21 (YSI 600QS Sonde and 650MSD handheld meter; YSI Inc., Yellow Springs, OH, USA).

Post-exposure measures

Fish were exposed for 21 days. Following exposure, all fish were weighed, and nine were randomly selected for immediate dissection. The remaining fish were transferred into clean 15-L aquaria ("group" tanks). Feeding was reduced to 3 ml melted FBS twice daily, flow rates were reduced to 40-60 ml/min per tank, and temperature was maintained near 24°C.

For dissections, fish were first anesthetized in clove oil (30 µl/l), weighed, measured for total length, and assessed for secondary sex characteristics. Ovipositor lengths and widths were measured in females and the number of tubercles were counted when present using a dissecting microscope. Banding and dorsal fatpads were graded using a scoring system of 0-4, where 0 = absence of the trait and 4 = very strong presence of the trait. Fish were euthanized by spinal severance, and gonads and livers were excised and weighed. Plasma and gonads were preserved at -80°C for determination of sex steroids and vitellogenin.

Post-exposure reproductive assessment

Three breeding substrates ("tiles"; half-sections of PVC drain tile) were placed in each group tank, and tiles were checked daily for eggs. Not all fish were sexually mature following cessation of exposure, and less than half displayed secondary sex characteristics. However, a variable number of fish within each tank clearly were reproductively mature and eggs were found in most tanks by day 4 post-exposure. Therefore, a subset of fish was used to form two breeding pairs per replicate on day 5 post-exposure as follows: first, additional 15-L aquaria were set up identical to the group tank except the newer tanks were divided in half using a perforated black acrylic divider. A single tank was assigned to each replicate, and positioning of tanks was randomized with respect to treatments. Next, all fish from a given replicate were removed and individual fish were placed in separate 1-L beakers filled with dechlorinated tap water. Each fish was weighed and identified as male, female, or immature/unknown based on secondary sex characteristics. All immature fish were returned to their original tank. Next, females and males were randomly assigned such that one male and one female were added to each half of a single "breeding" tank, and all remaining fish were returned to their original "group" tank. Fish returned to group tanks were allowed to mature 10 more days before being reassessed and divided into additional breeding pairs. The maximum number of male/female pairs was formed from each replicate, being limited by the number of males or females in each replicate.

Tiles were checked daily for eggs, and all tiles containing eggs were removed and immediately replaced with clean tiles. Eggs were carefully rolled from tiles, counted and assessed for fertility and viability. Removal of eggs from tiles was done under a dissecting microscope, and embryo development stages were determined using published descriptions (USEPA 1996). Unfertilized eggs were identified based on their distinct morphology, having a large translucent yolk mass and lacking the perivitelline space (USEPA 1996). Those eggs were delicate and often ruptured while being rolled from the tiles. By contrast, fertilized eggs have a well defined yolk mass, and a large perivitelline space which separated the yolk from the chorion. Eggs were considered viable if they contained a developing embryo. It was observed in previous studies that most eggs developed to around the gastrulation stage (stage 12, approximately 6 hours post-fertilization, hpf), after which a variable number would cease to progress. As such, all eggs were retained on tiles in a separate tank overnight, being inspected around stages 18 (formation of optic vesicles, 17-hpf) to stage 22 (first movements, 26-hpf). By this time, non-viable eggs were easily distinguished, most having a mottled yolk with no apparent embryo, or were opaque in colour.

One-hundred viable eggs, or all viable eggs when less than 100 were found, were incubated at 25°C in egg hatching cups in separate tanks. Fry were assessed following yolk-sac absorption at 7 - 8 days post-fertilization. Viable fry, dead fry, dead eggs, and abnormal fry were examined and counted under a dissecting microscope, and common physical abnormalities (yolk-sac and pericardial edema, spinal and craniofacial deformities, microphthalmia, anophthalmia) were recorded. Fry were preserved in absolute ethanol and stored at room temperature until DNA analysis.

Breeding pairs were arbitrarily assigned an identification code in order to conceal treatment history. All reproductive assessments were thus made blind to the treatments. Furthermore, all assessments of eggs, fry, and adults were made by a single observer (JLM), eliminating any observer-to-observer variability.

Fish were allowed to breed until day 42 post-exposure, after which all fish were euthanized and dissected as described above. White muscle, liver, and eye tissues were preserved in absolute ethanol and stored at room temperature until DNA analysis

Germline mutation assessment

Offspring from all breeding pairs were used to assess germ-line mutation rates. Twenty-two fry were randomly selected and processed from the first clutch produced by each pair. All fry were used when less than 22 fry were preserved for a given clutch (5 cases).

Germ-line mutations were identified as allele sizes in offspring that were not consistent with their parental genotypes. Four microsatellite loci (Table 3.2) reported to have a high number of alleles and high heterozygosities (Ardren et al., 2002; Bessert and Orti, 2003) were selected for analysis. Mutations were considered to have occurred when an allele size was at least 2 base-pairs larger or smaller than the corresponding parental allele.

Mutations were identified based on mismatched genotypes, in which one offspring allele most likely matched to one parent but the other allele did not match the other parent. PCR and size separation of the mismatched individual and both parents was re-performed to verify that mismatched alleles were not a result of PCR errors or variations in electrophoretic conditions. The progenitor allele was assumed to be the allele in the mismatched parent closest in size to the mutated allele.

All scoring of alleles was performed by the same observer (JLM). Each egg clutch was assigned a 3-digit identification number by chronological order among all clutches produced by all breeding pairs, irrespective of treatment or pair. These codes were used to identify all samples during molecular work and subsequent genotyping, effectively concealing treatment history during the mutation analysis. Clutches were linked to breeding pairs (which were also coded, details above), and breeding pairs were linked to treatments upon completion of the mutation analysis.

DNA isolation

All samples were soaked in ddH_2O for at least 6 hours (at room temperature) to a maximum of 24 hours (at 4°C) to remove ethanol from tissues prior to DNA isolation.

Adult white muscle (10-20 mg) was digested in 450 µl of lysis buffer (10 mM Tris [pH 8.0], 10 mM EDTA, 0.5% SDS, 100 mM NaCl, 500 µg/µl Proteinase-K) by incubating samples at 55°C for 18-24 hours with occasional vortexing. Next, 135 µl of 7.0 M ammonium acetate was added and samples were incubated on ice for 10 minutes followed by centrifugation 10 minutes at 14,000g to precipitate proteins. 500 µl of supernatant was transferred to new tubes and DNA was precipitated by adding 500 µl of isopropanol, followed by incubation at room temp for 15 minutes and centrifugation for 10 minutes at 14,000g. The isopropanol was then decanted and the DNA pellet was washed by adding 1000 µl of 70% ethanol, briefly and gently mixed, and a final centrifugation for 10 minutes at 14,000g. The final DNA pellet was air-dried and resuspended in 100 µl of TE buffer (10 mM Tris [pH 8.0], 1 mM EDTA).

DNA was extracted from fry following Neff et al. (2000). Briefly, whole fry (2-3 days post-hatch, yolk sacs absorbed) were incubated for 6-8 hours at 65°C in 50µl of TE buffer containing Proteinase-K (10mM Tris [pH 8.0], 1 mM EDTA, 500µg/µl Proteinase-K). Samples were heated to 95°C for 10 minutes and then centrifuged for 10 minutes at 14,000g to remove proteins and impurities. Supernatant was used directly for PCR amplifications.

PCR amplification

Unlabelled and fluorescently labeled primers were custom synthesized (Integrated DNA Technologies, Coralville, IA, USA) according to published primer sequences (Ardren et al., 2002; Bessert and Orti, 2003; Table 3.2). Two separation and visualization methods were used: automated capillary electrophoresis using fluorescent-labeled primers, and polyacrylamide gel electrophoresis using autoradiography (details below). PCR protocols varied depending on which method was used.

Method A: Polyacrylamide gel electrophoresis

Forward primers were radio-labeled by combining 1µl of forward primer (10 µM) with 0.33 µl of polynucleotide kinase (30U/µl; USB Corporation, Cleveland, OH, USA), 2 µl dilution buffer, 1µl PNK buffer (10x), 4.77 µl ddH2O, and 1µl γ-^{33}P (3000 Ci/mmol; Easytides ATP, PerkinElmer Life and Analytical Sciences, Woodbridge, ON, Canada), followed by incubation at 65°C for 30 minutes and then 95°C for 10 minutes

10-µl PCR reactions for the microsatellite loci *Ppr101* or *Ppr107* were 10 mM Tris-HCl, 50 mM KCl, 2.0 mM $MgCl_2$, 0.2 mM of each dNTP, 0.3 mg/ml BSA (Sigma – Aldrich, Oakville, ON, Canada), 0.5 µM unlabelled reverse primer, 0.46 µM unlabeled forward primer, 0.04 µM labeled forward primer, 0.4 units Taq (illustra rTaq; GE Healthcare Bio-Sciences Corp, Baie d'Urfe, QC, Canada), and 1µl of template DNA. PCR reaction for microsatellite loci *Ppro118* or Ppr132 were similar except for the following adjustments: 2.5 mM $MgCl_2$, 0.4 µM unlabelled reverse primer, 0.36 µM unlabelled forward primer, and 0.04 µM radio-labeled forward primer.

Microsatellite loci *Ppro118* and *Ppro132* were often co-amplified in 10-µl multiplex reactions that were identical to their single-locus reactions except that equal amounts of both primers were used (0.4 µM each reverse primer, 0.36 µM each unlabelled forward primer, and 0.04 µM each radio-labeled forward primer).

Amplification was carried out using either an Applied Biosystems Gene Amp 9700 or a MJ Research PTC-200 thermocycler set as follows: 95°C for 3 minutes, 35 cycles of [95°C for 30 seconds / 50°C (*Ppr101* and *Ppr107*) or 60°C (*Ppro118* or *Ppro132*) for 30 seconds / 72°C for 30 seconds], and a final elongation step of 72°C for 5 minutes. 3 µl stop solution (95% formamide, 0.1% bromophenol blue, 0.1% xylene cyanol, 10 mM EDTA) was added to all samples immediately following PCR. Samples were denatured at 95°C for 3 minutes and held on ice prior to being loaded onto pre-warmed 20 cm x 40 cm x 0.4 mm 6% denaturing polyacrylamide gels (OmniPur; EMD Chemicals Inc, Gibbstown, NJ, USA) containing 8.0M urea (JT Baker; Phillipsburg, NJ, USA). Samples were run in TBE buffer (89 mM Tris, 89 mM Boric Acid, 2 mM EDTA) for 3 or more hours at 50-100W which was adjusted to maintain a glass plate surface

temperature of 54-56°C. Following electrophoresis, gels were transferred to 0.35 mm chromatography paper (Fisher Scientific, Ottawa, ON, Canada) and dried at 80°C approximately 1 hour under vacuum (Bio-Rad gel dryer model 583; Bio-Rad Laboratories, Mississauga, ON, Canada). Storage phosphor screens were exposed to the dried gels overnight and were scanned using a Molecular Dynamics Storm 820 imaging system. Images were analyzed in ImageQuant version 5.2 (Molecular Dynamics Inc.), and allele sizes were determined by comparing alleles to a pUC19 DNA sequencing standard that was run on each gel (CycleReader DNA Sequencing Kit; Fermentas Life Sciences, Burlington, ON, Canada).

Method B: Capillary Electrophoresis

Capillary electrophoresis utilizing fluorescently-labeled forward primers used identical PCR conditions as above except that primer concentrations were reduced as follows. For amplification of *Ppr101* or *Ppr107*, 0.4 μM unlabelled reverse primer and 0.4 μM fluorescently-labeled forward primer was used. For amplification of *Ppro118* or *Ppro132*, 0.3 μM unlabelled reverse primer and 0.3 μM fluorescently-labeled forward primer was used. For multiplex reactions, 0.3 μM of each *Ppro118* primer and 0.2 μM of each *Ppro132* primer were used.

10-μl PCR products were desalted by precipitating in 31 μl 95% ethanol and 1 μl of 7.5M ammonium acetate, followed by a wash step using 150 μl 70% ethanol. Pellets were resuspended in 10 μl ddH2O. When PCR products were pooled prior to precipitation, all volumes above were doubled except for the 70% ethanol wash step.

Samples were size-separated on a MegaBACE 1000 (GE Healthcare Bio-Sciences Corp, Baie d'Urfe, QC, Canada) at the DNA and Forensic Sciences Centre, Trent University (Peterborough, ON, Canada). An internal size standard (MegaBACE ET550-R; GE Healthcare Bio-Sciences Corp, Baie d'Urfe, QC, Canada) was included in all samples. Allele sizes were determined using the Genetic Profiler 2.2 software.

Allele sizes determined using the MegaBACE system were often larger than when determined using polyacrylamide gel electrophoresis (PAGE). Alleles typically appeared 2 bps larger on the MegaBACE as compared to PAGE, but *Ppr107* and *Ppro132* alleles

under 170 bps were identical in size on both systems. Parental DNA served as positive controls on both systems, to allow for determination of variations in allele size estimations between the two systems. All allele sizes reported herein are as determined using PAGE.

Statistical Analysis

Nested analysis of variance was used to compare reproductive and physiological endpoints among treatments, with replicates (tanks or cages) nested within treatments. In the Nested ANOVA, variation among replicates was first tested ($F_{replicate} = MS_{replicates} / MS_{error}$). When no significant variation was found within replicates ($F_{replicates} < F_{critical}$), the F-statistic was calculated as $F_{treatment} = MS_{treatment} / MS_{error}$. When a significant variation was found among replicates, the F-statistic was adjusted to account for this variation ($F_{treatment} = MS_{treatment} / MS_{replicates}$).

All data were tested to ensure that the assumptions of the ANOVA were met (homogeneity of variance, residuals normally distributed, skewness and kurtosis within acceptable range) for each treatment group. When necessary, data were transformed using a logarithmic (base 10) or square-root transformation. Data that still failed to meet the assumptions even after trials with additional transformations were tested using the Kruskal-Wallis non-parametric analysis of variance. In these cases, each treatment group was first tested with the Kruskal-Wallis test to ensure that replicates within treatments were similar. In all such cases no significant differences were found, and all values were then pooled between replicates within treatments.

When a significant difference was found among treatments, means were compared using Tukey's all-pairwise comparisons (Nested ANOVA) or the Kruskal-Wallis all-pairwise comparison testing procedure provided in the Statistixs software.

Generalized estimating equations that fit the data to a Poisson regression while adjusting for multiple samples within families were used to compare the microsatellite mutations rates between treatments (Tsyusko et al., 2007). The input values for each individual is the mutation rate for that individual, calculated as the number of mutant bands divided by the total bands scored (two bands per loci x three loci included in the

analysis = six bands). Per-individual mutation rates were transformed using the improved angular transformation prior to analysis (Sokal and Rohlf, 1995). Fisher's Exact tests were also used to make pairwise comparisons of mutation rates between treatments.

Data exploration (testing for the assumptions of ANOVA) and contingency table testing were performed in Systat 11 (Systat Software Inc, Chicago, IL, USA). Generalized estimating equations, Fisher's Exact tests, and the power analysis of the Fisher's Exact tests were performed in SAS 9.1 (SAS Institute Inc, Cary, NC, USA). All other statistical calculations were performed using Statistix 9 (Analytical Software, Tallahassee, FL, USA). For all tests, α was set at 0.05. All data is present as the mean ± SEM (standard error of the mean) unless otherwise indicated.

RESULTS

Exposure period observations

Sediment tanks had reasonable water clarity prior to the addition of fish. No barriers were used between fish and sediment, and tanks became quite turbid within a short time following the addition of fish. Fish continuously stirred up the sediment in tanks, regardless of sediment type. Water clarity remained very poor for the duration of the exposure, but began to improve slightly toward the end of the exposure period. Initially, sediment was distributed in tank in a uniform layer across the entire bottom of the tanks. However, sediment in most tanks had accumulated into mounds at the front of tanks by the end of the exposure period, due to the combination of resuspension of sediments by fish and flow of water from the rear of the tank (water supply) to the front of the tank (water output). It was evident that some sediment was lost from tanks due to the flow of water through the tanks; however, the net loss of sediment over the exposure period was expected to be small, but was not determined.

Small oil-sheens were often seen on the surface of the Randle tanks during the exposure, and these tanks emitted a weak oil-grease odour that was not noted for LaSalle tanks. The composite Randle sediments also had a characteristic oil-grease odour at set-

up, and this odour was still notable when sediments were recovered from tanks following the exposure period.

Adults

Initial average weight of all fish was 1.21 grams (95% C.I. = 1.16 - 1.25; n = 264), and average weights within replicates were similar among treatments (Nested ANOVA, $F = 1.10$, $d.f. = 2,252$; $p = 0.333$). Average fish weight increased in all replicates, with similar weight gains being seen among treatments (Kruskal-Wallis, $F = 1.78$, $d.f. = 2,9$; $p = 0.224$; Figure 3.1), so that final fish weights did not differ among treatments (Nested ANOVA, $F = 0.43$, $d.f. = 2,249$; $p = 0.648$).

Survival was high in all treatments, with only two mortalities being observed: one in a LaSalle tank and the other in Randle tank. One fish was unaccounted for in another Randle tank at the end of the exposure; that fish may have died and become covered in sediment although a search of the sediment following the exposure period failed to return a carcass.

Subsets of nine randomly-selected fish per replicate were dissected immediately after the exposure. All fish were mature enough to allow for determination of gender based on examination of the gonads and secondary sex characteristics.

For females, LSI scores differed significantly among treatments (Nested ANOVA, $F = 5.85$, $d.f. = 2,58$; $p = 0.005$; Table 3.3); Randle and LaSalle females had similar LSI scores (Tukey's, $p > 0.05$) and both were larger than the controls (Tukey's, $p < 0.05$; Table 3.3)

Female GSI and female K scores did not differ among treatments (Nested ANOVA, $F = 1.03$, $d.f. = 2,57$; $p = 0.362$ and $F = 1.20$, $d.f. = 2,9$; $p = 0.346$ respectively; Table 3.3). In males, both LSI and K scores differed among treatments (Kruskal-Wallis, $F = 4.45$, $d.f. = 2,33$; $p = 0.020$ and $F = 6.71$, $d.f. = 2,33$; $p = 0.004$ respectively; Table 3.3), with no difference in GSI scores being observed (Nested ANOVA, $F = 0.07$, $d.f. = 2,24$; $p = 0.930$; Table 3.3). However, post-hoc analysis of the male LSI values failed to detect a significant difference between any pair of treatment means (Tukey's, $p > 0.05$).

This may be due to a high degree of variance, particularly within the Randle treatment (mean ± s.d: control = 1.91 ± 0.41; LaSalle = 2.36 ± 0.34; Randle = 2.70 ± 0.94).

Although secondary sex characteristics could be assessed in most fish, no significant differences were found among treatments for ovipositor length and width in females, and for dorsal fatpad score and number of nuptial tubercles in males (Table 3.3). Bands were absent on all males so no comparisons could be made. Furthermore, no males were observed to exhibit any female secondary sex characteristics, nor did any females exhibit male characteristics.

Post-exposure reproductive assessment

Of 57 pairs formed, 45 (79%) were successful in producing at least one clutch of eggs. Breeding success was highest in Randle pairs (17 of 18 pairs, 94% success), and lowest in control pairs (13 of 21 pairs, 62%). LaSalle was intermediate with 15 of 19 pairs producing eggs (79%). The distribution of successful breeding pairs within treatments was almost significantly different ($\chi^2 = 5.93$, $d.f. = 2$; $p = 0.052$).

A number of fish died or became visibly ill and had to be euthanized during the breeding period. Mortalities occurred in both breeding and non-breeding pairs. LaSalle pairs experienced the greatest number of mortalities, followed by control pairs. Only one Randle pair was affected by mortalities during the 42 day breeding period. The proportion of breeding fish that died in each replicate was not significantly different among treatments (Kruskal-Wallis, $F = 2.60$, $d.f. = 2,9$; $p = 0.128$).

Since reproduction in many pairs was interrupted due to the loss of one member, egg production rates were adjusted by dividing total egg production over the number of days between pair formation and pair termination due to death of one member or due to completion of the breeding period. The standardized egg production rates were significantly different among treatments (Nested ANOVA, $F = 5.43$, $d.f. = 2,34$; $p = 0.009$), with LaSalle and Randle pairs exhibiting similar rates (Tukey's, $p > 0.05$) and both groups producing significantly more eggs/day than control pairs (Tukey's, $p < 0.05$; Fig LS_EGGS). Average clutch size also differed among treatments (Nested ANOVA, F

= 4.36, *d.f.* = 2,34; $p = 0.021$), with LaSalle clutches being significantly larger than those produced by control pairs (Tukey's, $p < 0.05$; Figure 3.2).

Egg fertility rates were high and did not differ among treatments (Kruskal-Wallis, $F = 0.59$, *d.f.* = 2,42; $p = 0.561$; Figure 3.3). Egg viability rates were more variable and differed significantly among treatments (Kruskal-Wallis, $F = 8.65$, *d.f.* = 2,42; $p = 0.0007$; Figure 3.3). Egg viability rates in Randle pairs was slightly higher than in control pairs, but the difference was not significant (Kruskal-Wallis all-pairwise comparison test, $p > 0.05$). Contrarily, viability rates among LaSalle pairs were significantly higher than those in either control or Randle pairs (Kruskal-Wallis all-pairwise comparison test, $p < 0.05$). Hatching success rates were high in all treatments, and the percentage of unhacthed eggs did not differ significantly among treatments (Kruskal-Wallis, $F = 0.93$, *d.f.* = 2,42; $p = 0.404$; Fig 3.3). Few dead fry were present at enumeration (24-48 hours post-hatch) and no difference in fry mortality was observed among treatments (Kruskal-Wallis, $F = 0.62$, *d.f.* = 2,42; $p = 0.530$; Figure 3.3).

Approximately 1.4% of LaSalle fry and 1.8% of Randle fry were abnormal, whereas 3.0% of all control fry were abnormal; however, the fry abnormality rates were not statistically different among treatments (Kruskal-Wallis, $F = 0.97$, *d.f.* = 2,42; $p = 0.388$, Figure 3.3). Yolk-sac and pericardial edema were the most commonly observed abnormalities, each occurring in 80% of abnormal fry. Other common defects included spinal and craniofacial deformities (57% and 49%), microphthalmia (one or both eyes smaller than typical; 38%), and anophthalmia (one or both eyes missing; 3%). Most abnormal fry (79%) had two or more defects. Movement was considered highly abnormal in 91% of all abnormal fry and moderately abnormal in another 7%. These fry often displayed a limited and highly erratic pattern of movement (random or sporadic twitching, typically only able to swim in circles or with no clear direction). Such abnormalities would likely be lethal within a short time under natural conditions due to starvation (inability to seek out food) or predation (inability to avoid predators). It should be noted, however, that fry were not individually examined for abnormalities, but instead any fry exhibiting abnormal behaviour or movement were isolated and examined in more

detail. As such, selection bias towards fry with more extreme abnormalities likely occurred, and others with much less pronounced abnormalities may have been overlooked. Nevertheless, I believe that those numbers are a reasonable estimate of the number of fry that were adversely affected by physical abnormalities.

Following the 42 day breeding period, all fish were euthanized and dissected. No treatment effects were observed for any endpoint measured in males or females (Table 3.4), although two endpoints (number of tubercles and dorsal fatpad index in males) were almost significantly different among treatments (Nested ANOVA, $F = 3.06$, $d.f. = 2,45$; $p = 0.057$ and $F = 2.93$, $d.f. = 2,45$; $p = 0.064$, respectively). Post-hoc analysis of those data also showed that no significant differences in treatment mean values existed for any pairwise comparison (Tukey's, $p > 0.05$).

Germline mutation assessment

In total, 1080 offspring from 50 breeding pairs were analyzed at 4 microsatellite loci. A total of 8 mutant alleles were identified, all within the locus *Ppro118* (Table 3.5). Confident assignment of parental origin was possible for all mutant alleles, due to sufficient allelic diversity for *Ppro118* among breeding individuals. Additionally, all offspring carrying a mutated *Ppro118* allele matched both parents for the other three loci.

Mutation rates for *Ppro118* and overall mutation rates as well as their 95% confidence intervals were calculated for each treatment and also for all individuals combined (Table 3.6). Although the majority of mutations were observed in offspring originating in Randle-exposed fish, the difference in mutation rates among treatments was not statistically significant when testing using the generalized estimating equations ($\chi^2 = 4.20$, $d.f. = 2$; $p = 0.123$). Similar results were obtained when the data was tested using Fisher's Exact tests (Table 3.7). Unfortunately, the Fishers Exact tests had low power for detecting a significant result if one truly existed (β) given the sample sizes used and proportion of mutated bands observed (Table 3.7).

In Randle clutch #532, three individuals shared an identical maternally-derived mutant allele, and another two individuals shared an identical paternally-derived mutant allele (Table 3.5). While these may be the result of unique mutations events, they are

more likely the result of pre-meiotic mutations which gave rise to multiple gametes possessing the same mutant allele (Jones et al., 1999; Steinberg et al., 2002). Representative gels showing mutated and non-mutated alleles are shown in Appendix 1.

In all cases, mutated alleles originated through the addition or loss of one repeat unit (4 bps). Additions occurred in at least 7 of the 8 cases. In the 8th case, the two paternal alleles differed by 8 bps, with the mutated allele falling exactly halfway between the two and therefore may have been produced by the addition of 4 bps to the smaller paternal allele or by the loss of 4 bps from the larger paternal allele (Table 3.5).

Null alleles (alleles that do not amplify due to sequence variations within either or both of the primer binding sites) were identified at Ppr101 and Ppr107, based on comparison of amplifiable allele patterns in offspring and parents. Null *Ppr107* alleles were present in many individuals. Positively identifying null *Ppr107* alleles in adults and offspring was complicated by low allelic diversity for *Ppr107*. Of the nine allele sizes encountered, four (155, 164, 175, and 200 bps) accounted for 89% of all amplifying parental alleles. Identifying the presence of null alleles was simplified when parents did not share amplifying alleles. In several cases, noncongruence in banding pattern between adults and offspring suggested the presence of null alleles, but the parental source of the null could not be determined since both parents had identical-sized amplifiable alleles. In extreme cases, both parents were homozygous for null alleles (one case), or one parent was homozygous and the other adult carried a null allele and an amplifiable allele (at least one case). In the former, no amplifying bands were encountered. In the latter, approximately half of the offspring showed one band, and the rest did not show any amplifying bands. Of 100 breeding adults, at least 30 individual were heterozygous carriers of a *Ppr107* null allele (18 males, 10 females, 2 ambiguous), and at least 4 were homozygous carriers (3 males, 1 female).

Of the 2160 offspring alleles for *Ppr107*, 450 null alleles were strongly suspected (based on allele drop-out patterns). This is an underestimation of the total number of null alleles, since offspring potentially carrying a null allele (one parent is a carrier) could not be distinguished from offspring which were homozygous for an amplifying allele. Such a

case might occur when one parent carried a null allele and shared its amplifying allele with the other parent.

Mutation rates were not calculated for *Ppr107*, given the frequent occurrence of null alleles. Data for this locus were also excluded when calculating locus-specific and overall mutation rates.

Null *Ppr101* alleles were rarely encountered, with 5% of adults carrying single copies of a null allele. Data for this locus were also excluded for families in which one or both parents were suspected of carrying a null allele. Null alleles were not encountered in *Ppro118* or *Ppro132*.

Allele sizes within each microsatellite locus were similar to what has been reported by others (Ardren et al., 2002; Bessert and Orti, 2003; Bessert et al., 2007). For *Ppr101*, alleles ranged from 218 bps to 252 bps. For *Ppr107*, alleles ranged from 155 – 200 bps, with one additional allele of 287 bps. For *Ppro118*, most alleles occurred between 200 and 271 bps, with additional alleles between 396 and 408 bps. For *Ppro132*, alleles were found between 127 and 177 bps.

Chapter 4: Discussion

There is little evidence to suggest that fish exposed to Randle Reef sediments were adversely affected when compared to either the LaSalle reference group or the unexposed control group. Of all health and reproductive endpoints measured, Randle-exposed fish were statistically similar to either or both of the reference and control groups. Whenever Randle-exposed fish differed significantly from the unexposed control fish, the mean endpoints suggested that the Randle-exposed fish were in better health than the control fish.

Survival of adults was high in both the field and laboratory experiments. That was expected, since adult fish are considered to be relatively resistant to many contaminants that are acutely lethal to the embryo-larval stages (Nebeker et al., 1974; Burton, 1992). High adult survival was desirable in the present study in order to have sufficient numbers for the post-exposure breeding assessments. The development of a novel technique for the *in-situ* exposure of fish for relatively long periods prevented the high mortality rates that were encountered in a preliminary exposure trial (Jason Miller, unpublished data). A more detailed discussion of that caging technique is presented in Appendix Three.

Exposure to contaminated sediments did not appear to affect the overall heath status of adult fish. Body weight, GSI, LSI, and *K* values were similar among treatments at each assessment, with a few exceptions. Following the *in-situ* exposure, females exposed in the field were remarkably larger than control females, and also had larger gonads relative to body weights. In that experiment, I anticipated that a portion of the food would be lost from the field cages, and so provided twice as much food in order to compensate for the loss. That likely explains the larger size of Randle and LaSalle females, but doesn't fully explain why GSI was remarkably lower in control females. Alternatively, it is possible that the field fish received environmental cues that encouraged sexual maturation, leading to increased ovarian development in females and concomitant increase in body weight. Interestingly, the onset time of egg production was similar in all groups, with the control group eggs being found on day 33 post-exposure

followed by Randle and LaSalle group eggs on day 37 post-exposure. A similar lack of effect on male GSI was reported in American plaice (*Hippoglossoides platessoides*) after exposure to PAH-contaminated sediments (Nagler and Cyr, 1997).

LSI was also elevated in fish exposed to sediment, but only in fish exposed in-lab. Randle females had significantly elevated LSIs relative to controls, but were similar to LaSalle females. Randle males also had the highest LSIs among the three treatments, followed by LaSalle and then controls. Although the difference in males LSI values was significant when tested in the Nested ANOVA, post-hoc testing could not distinguish among treatment groups, suggesting that any pairwise difference was small or that variance was high within treatments. LSIs were similar among lab-exposed treatment groups following the 42-day breeding assessment, which suggests that a sediment component common to both field sites was responsible for the elevated LSIs. Surprisingly, no difference in LSIs was observed in male American plaice (*Hippoglossoides platessoides*) following a five-month exposure to sediments from three contaminated sites, although the total PAH concentrations varied by 1-2 orders of magnitude among sites (Nagler and Cyr, 1997; Lee et al., 1999). Conversely, elevated LSI has often been reported in fish exposed to pulp mill effluents (Parrott et al., 2003; Rickwood et al., 2006), municipal wastewater effluents (Barber et al., 2007), and synthetic estrogens (Parrott and Blunt, 2005), although dose-response relationships have not always been as expected. Surprisingly, LSI does not appear to be a commonly measured endpoint in fish following PAH exposure, as additional examples were not forthcoming in the literature.

Reproductive performance of exposed adults was also similar among treatments in both experiments, but again with a few exceptions. Egg production was significantly different among treatments following the six-week field exposure, and fecundity also differed following the three-week lab exposure. In all cases, the difference appeared to be due to poor reproductive performance by control fish, as fecundity rates in Randle females were similar to rates in LaSalle females, and both were similar to baseline fecundity rates reported for fathead minnow (Watanabe et al., 2007). There are

conflicting reports in regards to the effects of PAH-exposure on egg production, with significantly reduced fecundity being reported in mummichog (*Fundulus heteroclitus*) following exposure to PAH-contaminated sediments relative to fish exposed to reference sediments (Nye et al., 2007), and no effect on egg production being reported for fathead minnows exposed to benzo[a]pyrene (White et al., 1999).

No treatment effects were observed for egg hatch rates, fry viability, and fry abnormalities. By comparison, significantly reduced hatch rates were observed in American plaice (*Hippoglossoides platessoides*) following exposure of males to PAH-contaminated sediment for five months (Nagler and Cyr, 1997). However, that study differed from the present one, in that only male fish were exposed, and eggs were artificially inseminated. Furthermore, the eggs were from a single female, which may have hidden the degree of variability that would have been encountered had multiple females been used.

The absence of adverse reproductive effects suggests that multigenerational effects were unlikely to occur following PAH exposure. However, the possibility remains that effects may not be revealed until the second generation. For example, White et al. (1999) who exposed 21-day old fathead minnow to benzo[a]pyrene for four months, measured effects in the unexposed F_1 and F_2 offspring, including decreased egg production by F_1 generation and decreased hatch rates and larval survival in F_2 generation. However, the exposed F_0 population showed only a small and non-significant decline in egg production and egg hatch rates relative to controls.

Using a slightly differing study design, Hall and Oris (1991) exposed mature fathead minnows to anthracene for up to nine weeks, with breeding. Eggs were collected daily and incubated in clean water. They reported significantly elevated levels of anthracene in the gonads of exposed fish, as well as in the produced eggs. However, egg production, egg hatching rates, fry survival, and fry abnormality rates were largely unaffected by the treatments except for a significant decrease in fry survival at the highest concentration. It should be noted that significant effects were found in that study when embryos were subsequently incubated in the presence of solar ultraviolet radiation, likely

due to photoactivation of anthracene into a more toxic derivative. Photoactivation of PAHs is not likely a concern at Randle Reef due to the depth at which the contaminated sediment lie as well as the general turbidity of the water (Jason Miller, personal observations; McDonald and Chapman, 2002). In the lab I did not measure the UV intensities emitted from the lighting sources as used and cannot determine whether photoactivation of PAH's might have occurred in the exposure aquaria. Photoactivation of PAH's has been reported in cell cultures exposed to individual PAH's under standard fluorescent tube lighting (Platt et al., 2008). A more comparable investigation, in which benthic invertebrates were exposed in-lab to PAH-contaminated sediments, suggests that the amount of UV-light emitted by standard fluorescent tube lighting in not sufficient to lead to enhanced toxicity through photoactivation of PAH's (Ankley et al., 1994). In that study, the intensity of UV-B was below the detection limit of the equipment used and the intensity of UV-A was approximately one-order of magnitude lower than recorded for a UV-light source, and two-orders of magnitude lower than recorded for natural sunlight measured outdoors at noon in mid-July (Ankley et al., 1994).

Besides the foregoing two studies (Hall and Oris, 1991; White et al., 1999), no other relevant investigations into multigenerational PAH-induced effects in fish that measured similar endpoints to the present study are available. A somewhat related inquiry into multigenerational effects following PAH exposure examined the heritable resistance to PAH-induced embryo mortality seen in a population of mummichogs (*Fundulus heteroclitus*) collected from a site with high concentrations of PAH contamination (Ownby et al., 2002). However, in that study the embryos were directly exposed to contaminated sediments, with prevalence of cardiac defects in the offspring being the only endpoint reported.

To what extend feeding, handling, and environmental conditions such as water turbidity affected the outcome is difficult to determine

A number of factors varied between treatments, and may have had some influence on the results. These include feeding rates during the field study, handing of fish or fish cages during the field study, and water turbidity during the lab study. Fathead minnow

are considered hardy against handling stress, and fish that were mildly anesthetized and handled once a week for three weeks had GSI scores and fecundity, egg hatch and embryo mortality rates that were equivalent to unhandled control fish (Kahl et al., 2001). Fish that are fed at a higher rate will be larger (Smith et al., 1978), as were the field-caged fish with supplemented food in my study. The nutritional status of an individual also affects toxicological susceptibility (Lanno et al., 1989), and the increased food provided to the fish caged at Randle Reef may have been a factor which contributed to the lack of observable response in those fish relative to the lesser-fed control fish. Furthermore, the provision of food in the field may have reduced or eliminated the amount of naturally available food eaten by the fish caged at Randle Reef. If intake of contaminated food is an important route of PAH uptake, then the true effect of exposure at this location may have been confounded by providing excessive amounts of laboratory-quality food.

Water clarity may have affected the degree to which the lab control fish were stressed. During the lab exposure, tanks that contained sediment were extremely turbid, and fish were not visible within the tanks. Therefore, anyone working in close proximity to the tanks would not have been as noticeable to the fish in these tanks, but would have been obvious to the control fish that were maintained in clean, sediment-free tanks. It may be that the control fish were more stressed by the presence of technicians than were the fish in the sediment tanks, which may explain the poorer condition and subsequently lower reproductive output of these fish.

Only a slight and non-significant elevation of microsatellite DNA germline mutations rates was observed in fish exposed in-lab to Randle Reef sediment, relative to controls. This is the first study to report tandem-repeat DNA mutation rates in fish following exposure to environmental contaminants. Elevated germline mutations rates have been reported in herring gulls (*Larus argentatus*) exposed to unidentified environmental contaminants in the Hamilton Harbour region (Yauk and Quinn, 1996). A follow-up study revealed that mutations rates were correlated to distance between breeding sites and nearby steel mills (Yauk et al., 2000). Mice held downwind of the steel

mills which are located on the shore of Hamilton Harbour had elevated germline mutation rates as a result of exposure to particulate-bound contaminants (Somers et al., 2002, 2004). Mice fed fish collected from Hamilton Harbour had germline mutation rates that were elevated although not significantly different from mice fed fish collected from a less-contaminated location, although the lack of significance may have been due to a reduced sample size and not necessarily due to a lack of effect (Somers et al., 2008).

However, mutation rates have not always matched expectations. For example, tree swallows (*Tachycineta bicolor*) environmentally exposed to PCB's did not exhibit elevated rates of minisatellite mutations, in spite of high PCB body burdens and laboratory evidence that linked PCB exposure to increased minisatellite mutation rates (Hedenskog et al., 1997; Stapleton et al., 2001).

Several dissimilarities between the gull and mouse studies and the present study may help explain the differing results. First, the exposure route was different. In the present study, fish were presumably exposed to contaminant via direct uptake from the sediment or the water, and likely also through ingestion of contaminated food or sediment particles. In the gull studies, the route of exposure was not identified but was suggested to be via airborne contaminants given the correlation between mutations rates and distance from steel mills. However, dietary exposure was not ruled out. Herring gulls are predominantly fish eaters and birds inhabiting the Hamilton Harbour region likely consume fish with elevated body burdens of many waterborne or sediment-bound contaminants (Yauk and Quinn, 1996; Yauk et al., 2000).

Second, different tandem repeat markers were used. The present study used microsatellite DNA markers, whereas the other studies and in fact all previous investigations into chemical mutagenesis in tandem repeat markers used minisatellites and expanded-simple-tandem-repeat (ESTR) DNA markers. Typically, higher mutation rates have been reported for minisatellites and ESTR's than for microsatellites. In respect to the reported spontaneous germline mutation rates for microsatellites, minisatellites, and ESTR's, one can generalize that most microsatellite loci have overall lower spontaneous mutation rates compared to minisatellites and ESTR's (Table 4.1). However

a few hyper-variable microsatellite loci have been reported with spontaneous mutation rates that approach those of the other markers (Table 4.1). That factor alone may explain why mutation rates were elevated in the gull and mouse studies but not in the present study. A more detailed examination of the advantages and disadvantages of various tandem repeat markers is presented in Appendix Four.

Third, detailed information of spermatogenesis in mice allowed for a very specific protocol to be established with respect to timing of exposure and analysis of offspring. In mice, a variety of mutagenic chemicals exert effects on male germ cells only if exposed at specific stages of spermatogenesis (reviewed by Singer et al., 2006). Typically, exposure of spermatogonial cells is required to induce elevated mutation rates, and thus the mice were not mated until sufficient time had elapsed to allow for maturation of the exposed spermatogonia. In mice, pre-lepotene spermatogonia mature to late spermatids in 41 days (see Singer et al, 2006). Although stages of spermatogenesis in fathead minnow have been described in detail (Smith, 1978; Jensen et al., 2001; van Aerle et al., 2004), the rate at which spermatogenesis proceeds remains to be determined. The limited published data suggest that spermatogenesis in fish proceeds quicker than in mammals, occurring within 10 –12 days in several freshwater teleost fishes (Egami and Hyodo-Taguchi, 1967; Sinha et al., 1979, 1982, 1983; Vilela et al., 2003). Durations up to 21 days have also been reported for other teleost species (see Vilela et al., 2003). Another important difference in fish is that the rate of spermatogenesis is strongly affected by temperature, with lower temperatures slowing or even inhibiting the maturation process (Egami and Hyodo-Taguchi, 1967; Vilela et al., 2003). Given the foregoing information, I assumed that the rate of spermatogenesis in fathead minnow maintained near 25°C would be equal to or less than the maximum rate reported in fish (21 days). For this reason, fish were exposed in-lab for 21 days. As such, the exposure duration was likely sufficient such that offspring were produced from spermatozoa exposed at the spermatogonial stages. Still, better characterization of spermatogenesis in fathead minnow may allow for more effective and informative study designs intended to evaluate

not only germline mutations but a host of biological effects that may occur though exposure of particular stages germ cells or gametes.

Yet another difference between studies is that the gull and mouse studies used tandem repeat loci that had already been characterized as hypermutable, whereas the present study used loci that had not been previously evaluated for mutatability. I selected microsatellite loci reported to have the greatest number of alleles and highest levels of heterozygosity, assuming that such characteristics reflect a greater propensity for mutations at those loci. Others have suggested that particular characteristics such as mean allele length, repeat unit length, and repeat purity may affect locus-specific mutation rates (Brinkmann et al., 1998; Schlotterer et al., 1998; Xu et al., 2000; Eckert et al., 2002; Lai and Sun, 2003). However, even a general examination of microsatellite mutations reported thus far indicates that those factors alone do not fully explain the variations in rates observed between tandem repeat loci. For example, examination of the mutational spectra seen in radiation-exposed and unexposed medaka (*Oryzias latipes*) reveal that two very similar pure repeats with tetrameric motifs, $(AGAT)_{25}$ and $(AACT)_{16}$, had the highest overall mutations rates whereas loci with longer and more complicated repeat motifs produced fewer mutations (Tsyusko et al., 2007). Clearly much work remains before we can better understand why mutations occur more frequently at some loci and rather infrequently in others.

While the sample sizes used resulted in low power for detecting a significant increase in mutation rates if one truly existed, the sample sizes that would be required to provide a β-value of 0.80 range from 771 per group for the comparison of mutation rates between Randle and LaSalle, to 2373 individuals per group for the comparison of mutation rates between Randle and controls. The large difference between required sample sizes for testing of Randle with LaSalle vs. Randle and controls is surprising, given that the LaSalle and control groups only differed by a single mutation.

Software calculating the power analysis for generalized estimating equations is not currently available. To provide some indication of the effect of sample size on the outcome, I doubled and tripled the sample sizes, while maintaining the mutations rates

seen for each treatment. Mutation rates would have been significantly different ($\chi^2 = 8.39$, $d.f. = 2$; $p = 0.015$) had a sample that was twice as large been used, with the same mutation rates being observed (i.e. 2 within controls, 0 within LaSalle, and 8 within Randle). Had the size been tripled, the difference in resulting mutation rates would have been highly significant ($\chi^2 = 12.59$, $d.f. = 2$; $p = 0.002$).

In the absence of detailed background mutations rates, selection of loci for use in mutational studies will continue to be unreliable. However, even though certain characteristics such as longer average allele size, greater number of alleles, and repeat unit length do not always predict mutability, they are likely the most reliable criteria in selecting markers when mutation rates are unknown.

An empirical approach, used by Tsyusko et al. (2007), is to use a large array of microsatellite markers to first screen a number of control individuals. Based on the results of this pre-exposure assay, the markers displaying highest mutation rates are selected for use. That approach, however, when used with less-variable markers may fail to detect enough mutations unless a relatively large sample size is used. An expanded initial assessment population may be acceptable if those data could also be used as the control data. However, care must be taken to ensure that the control and exposed populations are sufficiently similar to allow for a valid comparison to be made.

I identified germline mutations in only one of the four selected loci. While it is disappointing that three loci failed to exhibit mutations, it is not completely unexpected given that most papers describing the occurrence of induced or spontaneous microsatellite mutations have also reported that a number, and often a majority, of loci showed no mutations (Table 4.1). That fact should not be overlooked when evaluating germline mutations, as overall mutation rates may be strongly influenced by the number of loci for which no mutations were identified. In the present study, the statistical analyses was based on overall mutation rates as that approach has been used by other researchers (Somers et al., 2004; Stapleton et al., 2001; Yauk et al., 2000). However, reassessment of the data using only the *Ppro118* locus-specific mutation rates did not alter the conclusions.

Another possible explanation for the lack of elevated mutations as well as the lack of adverse health and reproductive effects could be that the exposed fish were inadequately exposed to the contaminants. Unfortunately, I did not employ any biological or chemical indicators to determine the degree of PAH-exposure experienced by the adult fish. However, biochemical endpoints measured in juvenile rainbow trout (*Oncorhynchus mykiss*) exposed in-lab to the same Randle-Reef sediment samples suggest that the samples were indeed enriched in PAH's and that uptake of PAH's from the sediment should have occurred (Jim Sherry, unpublished data). Sediment samples were collected for PAH analysis at the time of sediment mixing, but these have yet to be analyzed. Although not a measure of bioavailability, sediment PAH concentrations would at least be suggestive of the degree to which the fish were exposed to PAH's in-lab.

Indirect evidence also suggests that the PAH's contained in the sediment would have been available to the fish caged at Randle Reef. For example, Mac et al. (1990) caged fathead minnows at a contaminated site in the Detroit River for 10 days, with cages positioned at the sediment surface (2.4 meters depth) and approximately 1.2 meters above the sediment surface. Fish mortality rates were 22% in the bottom cage, and 24% in the mid-water cage, compared to mortality rates of 5% (water column) and 12% (bottom) seen in fish exposed at a less contaminated site. Furthermore, 27.5% mortality occurred in fish exposed to the same sediment in-lab. Taken together, this suggests that the toxic components present in the test site sediments were bioavailable to fathead minnows both at and 1.2 meters above the sediment surface. In a similar study, Leadly et al. (1999) caged brown bullhead (*Ameiurus nebulosus*) at three sites in the Detroit River, of which one was considered highly contaminated, the other moderately contaminated, while the third served as a reference site. Two cages were used at each site, one with its bottom slightly buried in sediment to allow fish direct contact with the sediment, while the second cage was positioned 0.6 meters above the sediment surface. Fish were subsampled at regular intervals, and levels of biliary fluorescent aromatic hydrocarbons (FAC) were used to indicate the degree of exposure to PAH's. Although temporal variations in FAC levels were seen, response levels at each time point were of similar magnitude between

bottom and water-column cages. Also, FAC levels were reflective of PAH contamination levels at each site. Collectively, this study also supports the notion that direct sediment contact is not required for sufficient exposure to the PAH's present in contaminated sediment.

Whereas the field-exposed fish did not have access to the bottom sediment, the lab-exposure fish had full access to the sediments, and so presumably experienced a greater degree of PAH-exposure. This "full spectrum" of exposure routes is important, as conflicting reports have been given in respect to whether direct sediment contact was required to elicit effects under lab conditions (Roberts et al., 1989; Kocan et al., 1996; Vines et al., 2000).

Another concern in laboratory sediment testing is whether the concentration of contaminants in the exposure tanks may decrease over the duration of the exposure period due to water exchange, biotic or abiotic degradation, or other route of loss of the contaminants. In the present study, neither sediment nor waterborne PAH concentrations were determined before or after the exposure period, so I cannot say if such occurred. The observation of a characteristic oil-grease odour in Randle Reef sediments before and after the exposure, as well as in the Randle exposure tanks during the in-lab sediment test, suggests that at least some volatile PAH's or other hydrocarbons were lost through volatilization during the exposure. However, it is unlikely that the larger and less volatile PAH's such as benzo[a] pyrene and benzo(g,h,i)perylene experienced a similar loss. Furthermore, published reports indicate that sediment PAH concentrations do not decline significantly during studies lasting up to 5 weeks in duration (French et al., 1996; Kocan et al., 1996). Additionally, measures of biochemical response and mortality suggest that the toxicity of the sediment may remain constant or decreases only slightly throughout test periods of up to 5 months (Roberts et al., 1989; Nagler and Cyr, 1997). Fragoso et al. (2006) specifically tested the effects of water exchange on biological response to PAH contaminated sediments and concluded that water exchange does not preclude observing a toxic response when using highly contaminated sediments.

The lack of specific information on PAH concentrations at the cage depth (field study) or in the exposure tanks (lab study) is an important limitation of this study. Without it, I am limited to presuming that the level of PAH-exposure experienced by the fish exposed to Randle Reef sediments should have been adequate to alter the reproductive and genotoxic endpoints that I used. In the absence of actual PAH concentration, or biomarkers of PAH exposure such as induction of detoxification enzymes or body burdens of contaminants, I cannot rule out the possibility that the fish were not exposed to high levels of PAH's, due to poorly selected exposure sites or sediment collection sites, or other study design related variables.

In conclusion, I did not find evidence to suggest fish exposed to contaminated sediments at Randle Reef are likely to experience heritable reproductive effects or elevated germline mutation rates. However, it would be premature to conclude that sediment contamination at Randle Reef does not pose a significant risk to the reproductive and genetic health of fish inhabiting that region of the harbour given the limitations of this study.

Nevertheless, the study generated valuable information, including a novel method of exposing small fish *in-situ* for extended durations. Furthermore, to the best of my knowledge this is the first evaluation of microsatellite germline mutations in sentinel organisms following exposure to environmental contaminants. More robust study designs are recommended, including more comprehensive evaluations of PAH-exposures by means of biochemical analysis or similar techniques. Further refinement of the study design may provide a sensitive method for evaluating heritable effects following exposure to environmental contaminants. Additional testing using other persistent pollutants such as PCB's or heavy metals may provide additional insights into the usefulness of this approach for monitoring genotoxic environmental contaminants.

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Table 2.1: Water quality values for influent water used in both studies. Water samples were collected monthly and were analyzed by the National Laboratory for Environmental Testing (Burlington ON) using certified methods.

Parameter		Field Study (Ch. 2)			Lab Study (Ch. 3)		
		Average	St. Dev.	Max.	Average	St. Dev.	Max.
Alkalinity (as $CaCO_3$)	mg/L	78.5	2.90	81.6	80.7	0.60	81.1
Hardness (as $CaCO_3$)	mg/L	123	1.80	125	123	3.50	125
pH		7.56	0.29	8.14	7.8	0.12	7.88
Specific Conductivity	µS/cm	338	23.2	381	324	1.40	325
Dissolved Organic Carbon	mg/L	1.10	0.40	1.40	1.05	0.07	1.10
Dissolved Inorganic Carbon	mg/L	18.5	0.60	19.2	19.8	0.20	19.9
Free Ammonia	mg/L	0.102	0.087	0.239	0.051	0.06	0.093
Nitrate / Nitrite	mg/L	0.459	0.084	0.588	0.455	0.021	0.47
Total Phosphorus	mg/L	0.028	0.002	0.032	0.029	0.001	0.030
Calcium	mg/L	34.8	0.50	35.5	34.9	1.30	35.8
Magnesium	mg/L	8.59	0.13	8.71	8.63	0.07	8.68
Sodium	mg/L	14.1	0.40	14.7	14.7	0.10	14.8
Potassium	mg/L	7.41	5.23	15.6	3.35	2.37	5.00
Chlorine	mg/L	26.3	0.80	27.2	27.1	0.40	27.4
Fluoride	mg/L	0.568	0.032	0.6	0.57	0.028	0.59
Aluminum	µg/L	26.7	11.1	38.5	20.2	3.6	22.7
Cadmium	µg/L	0.004	0.002	0.005	0.011	0	0.011
Copper	µg/L	0.318	0.185	0.55	0.39	0.17	0.51
Iron	µg/L	4.10	1.30	5.60	2.30	0.00	2.30
Nickel	µg/L	0.685	0.284	1.26	0.71	0.00	0.71
Lead	µg/L	0.097	0.085	0.213	0.268	0.201	0.41
Selenium	µg/L	0.157	0.038	0.200	0.155	0.007	0.160
Zinc	µg/L	0.613	0.406	1.39	1.325	0.53	1.70

Table 2.2: Physiological measures on adult fathead minnow following the breeding assessment period. Fish were exposed *in-situ* for 42 days prior to breeding. Data represents the mean ± SEM values per treatment. Number of fish in parentheses. All data analyzed using Nested ANOVA. No significant difference were found among treatments for all endpoints.

			Females			
Endpoint	**Control**	**LaSalle**	**Randle**	***F***	***df***	***p***
Weight	2.01 ± 0.16 (3)	1.77 ± 0.11 (17)	2.09 ± 0.11 (12)	1.18	2,20	0.329
GSI	5.74 ± 2.01 (3)	9.96 ± 1.77 (17)	9.10 ± 1.88 (12)	0.33	2,20	0.725
LSI	1.88 ± 0.29 (3)	2.68 ± 0.16 (17)	2.81 ± 0.28 (12)	2.46	2,20	0.111
K	1.20 ± 0.12 (3)	1.09 ± 0.04 (17)	1.11 ± 0.02 (12)	0.46	2,20	0.638
			Males			
Endpoint	**Control**	**LaSalle**	**Randle**	***F***	***df***	***p***
Weight	3.32 ± 0.15 (3)	3.73 ± 0.16 (17)	3.71 ± 0.21 (15)	0.50	2,24	0.611
GSI	0.59 ± 0.18 (3)	1.04 ± 0.08 (17)	0.89 ± 0.11 (15)	2.13	2,24	0.140
LSI	2.68 ± 0.34 (3)	2.11 ± 0.10 (17)	1.99 ± 0.15 (15)	1.33	2,24	0.283
K	1.14 ± 0.10 (3)	1.32 ± 0.05 (17)	1.29 ± 0.04 (15)	3.09	2,24	0.064

Table 3.1: GPS co-ordinates for sediment collection points at Randle Reef (field study site) and near LaSalle Marina (field reference site). Approximatly eight litres of sediment were collected at each location. Coordinates are Universal Transverse Mercator, North American Datum 1983

Northing	Easting
Near LaSalle Marina, Hamilton Harbour	
4794975	594270
4794948	594240
4794827	594153
4795100	594420
Near Randle Reef, Hamilton Harbour	
4792112	594322
4791860	594682
4791512	594659
4791495	594655
4791779	594499
4792117	594571
4792004	594705

Note: Although sediment was collected at seven sites near LaSalla Marina, co-ordinates are only available for four sites due to equipment failure. The three unrecorded sites were within close proximity to the four sites reported

Table 3.2: Microsatellite marker primer sequences and other characteristics .

Locus*	Primer Sequence (5'→3')	Repeat motif	Size (bp)	k	HO
Ppr101 G73218	F: TCCTTGAAATCAGAGGACTG R: CGAAACATCAGTACGCAAGC	$(AC)_{11}$	211–258	12	0.75
Ppr107 G73223	F: TATGTGGTGGAATGGGAGTC R: GGCTGAGAGAATAAGCTTGC	$(ACAG)_8$	164–285	11	0.84
Ppro118 AY254352	F: CCGGATGCACTGGTGGAGAAAA R: CCAGCAATCATAGCAGGCAGGAAC	$(CTAT)_{11}(CTGT)_{15}$	175–339	31	0.90
Ppro132 AY254354	F: GCATTTCCTTTTGCTTGTAAGTCTCAA R: GGTTTAACCCGATCAATGGCTGTGC	$(CT)_{18}$	130–184	19	0.84

* GenBank Accession number listed below locus name. Allele sizes, number of alleles (k), and observed homozygosity (HO) values are from the original assessment in 48-50 individuals (Ardren et al., 2002; Bessert and Orti, 2003).

Table 3.3: Physiological measures on adult fathead minnow immediately following the 21-day in-lab exposure. Data represents the mean ± SEM values per treatment. Number of fish in parentheses. All data analyzed using Nested ANOVA except male LSI and K, which were analyzed using the non-parametric Kruskal-Wallis ANOVA. Differing letters following parentheses indicate groups that differed when tested using Tukey's or the Kruskal-Wallis all-pairwise comparisons ($p < 0.05$).

	Females					
Endpoint	**Control**	**LaSalle**	**Randle**	***F***	***df***	***p***
GSI	7.16 ± 1.31 (20)	8.06 ± 1.05 (23)	9.24 ± 1.17 (26)	1.03	2,57	0.362
LSI	2.19 ± 0.12 (21) **A**	2.82 ± 0.15 (23) **B**	2.91 ± 0.19 (26) **B**	5.85	2,58	**0.005**
K	0.95 ± 0.02 (21)	0.99 ± 0.02 (23)	1.01 ± 0.02 (26)	1.12	2,9	0.346
Ovi Length	16.48 ± 1.11 (21)	14.32 ± 0.89 (22)	11.96 ± 0.89 (23)	0.13	2,9	0.125
Ovi Width	4.86 ± 0.35 (21)	4.00 ± 0.42 (22)	4.22 ± 0.36 (23)	1.72	2,54	0.189
	Males					
Endpoint	**Control**	**LaSalle**	**Randle**	***F***	***df***	***p***
GSI	1.41 ± 0.29 (14)	1.06 ± 0.18 (12)	1.29 ± 0.26 (10)	0.07	2,24	0.930
LSI	1.90 ± 0.11 (14) **A**	2.36 ± 0.1 0 (12) **A**	2.70 ± 0.30 (10) **A**	4.45	2,35	**0.020**
K	0.92 ± 0.02 (14) **A**	1.00 ± 0.02 (12) **B**	0.99 ± 0.03 (10) **B**	6.71	2,35	**0.004**
Tubercles	0.21 ± 0.15 (14)	3.75 ± 1.81 (12)	4.60 ± 2.39 (10)	2.96	2,24	0.071
FatPad Index	0.32 ± 0.11 (14)	0.75 ± 0.12 (12)	0.30 ± 0.13 (10)	2.11	2,9	0.177

Table 3.4: Physiological measures on adult fathead minnow following the 42-day breeding period. Data represents the mean ± SEM values per treatment. Number of fish in parentheses. All data analyzed using Nested ANOVA except female LSI and ovi-width, which were analyzed using the non-parametric Kruskal-Wallis ANOVA. No significant difference were found amoung treatments for all enpoints.

	Females					
Endpoint	**Control**	**LaSalle**	**Randle**	***F***	***df***	***p***
GSI	15.14 ± 1.33 (13)	14.46 ± 1.21 (11)	13.58 ± 0.85 (21)	0.93	2,33	0.704
LSI	2.57 ± 0.13 (13)	2.95 ± 0.32 (11)	2.9 ± 0.13 (21)	0.97	2,42	0.387
K	1.08 ± 0.04 (13)	1.04 ± 0.03 (11)	1.06 ± 0.03 (21)	0.38	2,33	0.688
Ovi Length	17.3 ± 1.81 (10)	19.64 ± 1.58 (11)	18.52 ± 0.78 (21)	1.10	2,30	0.348
Ovi Width	10.4 ± 0.56 (10)	10.36 ± 0.93 (11)	9.9 ± 0.34 (21)	0.13	2,39	0.877
	Males					
Endpoint	**Control**	**LaSalle**	**Randle**	***F***	***df***	***p***
GSI	1.16 ± 0.07 (19)	1.4 ± 0.18 (15)	1.35 ± 0.07 (22)	1.15	2,44	0.327
LSI	2.68 ± 0.15 (19)	2.72 ± 0.16 (16)	2.6 ± 0.12 (22)	0.13	2,45	0.881
K	1.16 ± 0.03 (19)	1.21 ± 0.03 (16)	1.21 ± 0.03 (22)	1.07	2,45	0.353
Tubercles	14.11 ± 1.22 (19)	18.19 ± 1.16 (16)	17.14 ± 0.89 (22)	3.06	2,45	0.057
FatPad Index	1.34 ± 0.18 (19)	1.94 ± 0.19 (16)	1.41 ± 0.16 (22)	2.93	2,45	0.064
Band Index	0.47 ± 0.22 (19)	0.93 ± 0.33 (15)	1.09 ± 0.26 (22)	1.56	2,44	0.222

Table 3.5: Germline microsatellite mutations observed in fathead minnow families. Mutations were observed in the offspring of parents previously exposed to contaminated sediment (Randle) or no sediment (Control). Mutated alleles in offspring and parental progenitor alleles shown in bold. Allele sizes are in base-pairs. All mutations occured at the locus *Ppro118*.

Pair ID	Clucth ID	Parental Exposure	Paternal Alleles	Maternal Alleles	Offspring Alleles	Change	Origin
7A	514	Randle	200 / **207**	231 / 249	**211** / 231	+4 bps	Paternal
24B	532	Randle	**204** / 232	200 / 254	200 / **208**	+4 bps	Paternal
24B	532	Randle	**204** / 232	200 / 254	**208** / 254	+4 bps	Paternal
24B	532	Randle	204 / 232	200 / **254**	204 / **258**	+4 bps	Maternal
24B	532	Randle	204 / 232	200 / **254**	232 / **258**	+4 bps	Maternal
24B	532	Randle	204 / 232	200 / **254**	232 / **258**	+4 bps	Maternal
18A	570	Randle	236 / **271**	227 / 236	236 / **275**	+4 bps	Paternal
35B	577	Control	**244** / **252**	200 / 244	200 / **248**	±4bps	Paternal

Table 3.6: Locus-specific and overall mutation rates for fish in each treatment and combined. Data for *Ppr107* excluded due to excessive frequency of null alleles. Data for *Ppr101* excluded only from families which exhibited null alleles (n = 5). 95% confidence intervals calculated based on a Poisson distribution.

		Ppr101		
Exposure	**Control**	**LaSalle**	**Randle**	**Total**
No. Mutations	0	0	0	0
No. meioses scored	594	656	680	1930
No. families examined	14	15	16	45
		Ppro118		
Exposure	**Control**	**LaSalle**	**Randle**	**Total**
No. Mutations	1	0	4	5
No. meioses scored	634	700	798	2132
No. families examined	15	16	19	50
Mutation rate	1.57×10^{-3}	0	5.01×10^{-3}	3.75×10^{-3}
Lower 95% C.I.	3.99×10^{-5}	0	1.37×10^{-3}	0.76×10^{-3}
Upper 95% C.I.	8.79×10^{-3}	5.27×10^{-3}	1.28×10^{-2}	5.47×10^{-3}
		Ppro132		
Exposure	**Control**	**LaSalle**	**Randle**	**Total**
No. Mutations	0	0	0	0
No. meioses scored	638	702	802	2142
No. families examined	15	16	19	50
		Overall		
Exposure	**Control**	**LaSalle**	**Randle**	**Total**
No. Mutations	1	0	4	5
No. meioses scored	1866	2058	2280	6204
Mutation rate	0.54×10^{-3}	0	1.75×10^{-3}	0.81×10^{-3}
Lower 95% C.I.	1.36×10^{-5}	0	0.48×10^{-3}	0.26×10^{-3}
Upper 95% C.I.	2.99×10^{-3}	1.79×10^{-3}	4.49×10^{-3}	1.88×10^{-3}

Table 3.7: Results of the Fisher's Exact tests and subsequent power analysis. n = group size, m = number of mutations observed, r = proportion of individuals with a mutation, p = p-value of the Fisher's Exact test, β = power of the Fisher Exact test.

	Control n = 317 m = 1 r = 0.0032	**LaSalle** n = 350 m = 0 r = 0.0000*
LaSalle n = 350 m = 0 r = 0.0000*	p = 0.475 β = 0.050	
Randle n = 399 m = 4 r = 0.0100	p = 0.390 β = 0.100	p = 0.127 β = 0.322

* For the power analysis, a proportion of 0.000001 was used for LaSalle since the test would not run using a value of 0.0000

Table 4.1: Spontaneous mutation rates reported for three classes of tandem repeats. Number of loci tested, number of loci positive for mutations, and total number of mutations observed are as reported by the authors. Overall mean mutation rates and locus-specific mutation rates as reported or estimated where necessary. Only control group (unexposed) data included from induced mutation studies. Missing data indicates values that were neither reported nor could be estimated given the information supplied by the authors. Scientific names, references, and footnotes provided on next page.

Species or Common Name	Loci		Mutations observed	Mutation Rate (x 10^{-3})	
	tested	positive		overall[a]	locus-specific[b]
Microsatellites					
drosophila	24	1	9	0.0063	0.30
freshwater snail	11	4	10[c+]	1.05	1.16 - 5.79
common carp	49	3	3	0.56	9.10
japanese medaka	9 (27)[d]	7	16	8.89	5.00 - 10.00
pink salmon	9	2	16[c+]	1.37	3.90 - 8.50
pipefish	4	2	26[c-]		0.94 - 3.10
senegal sole	12	2	3	0.39	1.56 - 3.13
sea turtle	2	1	33	11.7	23.5
salamander	6	1	10	0.13	4.98
barn swallow	2	2	44	20.6	4.40 - 39.0
barn swallow	1	1	31	34.7	
barn swallow	3	3	53	18.1	
lesser kestral	9	4	10	2.96	2.78 - 8.68
swine	38	4	5	0.075	0.57 - 1.14
sheep	215	3	5[c+]	0.11	4.65 - 13.9
human	9	6	23	2.12	1.17 - 6.84
human	273		508	1.77	
humans	15	3	3	0.90	2.90 - 7.90
humans	72	15	21	5.78	7.40 - 29.4
humans	12	6	10	1.39	1.67 - 5.00
Minisatellites					
brown trout	11	0	0	0	0
herring gull	3		12	4.31	
herring gull	2	2	32	6.1	
tree swallow	1	1		11.45	
humans	6	5	13	5.3	2.1 - 19.4
Expanded Simple Tandem Repeats					
mouse	2	0	0	0	0
mouse	3	3	125	141.4	153.3 - 217.9
mouse	2	2			50 - 70
mouse	3	3	131	33.7	10.8 - 123.3
mouse	1			62.5	

Table 4.1 continued. Scientific names and references.

Species or Common Name	Scientific Name	References
	Microsatellites	
drosophila	*Drosophila melanogaster*	Schlotterer et al., 1998
freshwater snail	*Bulinus forskalii*	Gow et al., 2005
common carp	*Cyprinus carpio*	Yue et al., 2007
japanese medaka	*Oryzias latipes*	Tsyusko et al., 2007
pink salmon	*Oncorhyncus gorbuscha*	Steinberg et al., 2002
pipefish	*Syngnathus typhle*	Jones et al., 1999
senegal sole	*Solea senegalensis*	Castro et al., 2006
sea turtle	*Lepidochelys olivacea*	Hoekert et al., 2002
salamander	*Ambystoma tigrinum tigrinum*	Bulut et al., 2008
barn swallow	*Hirundo rustica*	Ellegren et al., 1997
barn swallow	*Hirundo rustica*	Brohede et al., 2004
barn swallow	*Hirundo rustica*	Brohede et al., 2004
lesser kestral	*Falco naumanni*	Ortego et al., 2008
swine	*Sus sp.*	Yue et al. 2002
sheep	*Ovis aries*	Crawford et al., 1996
human	*Homo sapian*	Brinkmann et al., 1998
human	*Homo sapian*	Xu et al., 2000
humans	*Homo sapian*	Farfan et al., 2003
humans	*Homo sapian*	Furitsu et al., 2005
humans	*Homo sapian*	da Cruz et al., 2008
	Minisatellites	
brown trout	*Salmo trutta*	Prodoh et al., 1994
herring gull	*Larus argentatus*	Yauk et al., 1996
herring gull	*Larus argentatus*	Yauk et al., 2000
tree swallow	*Tachycineta bicolor*	Stapleton et al., 2001
humans	*Salmo trutta*	Farfan et al., 2003
	Expanded Simple Tandem Repeats	
mouse	*Mus musculus*	Hedenskog et al., 1997
mouse	*Mus musculus*	Somers et al., 2002
mouse	*Mus musculus*	Vilariño-Güell et al., 2003
mouse	*Mus musculus*	Somers et al., 2004
mouse	*Mus musculus*	Glen et al., 2008

Notes: [a] Mean overall mutation rates = total number of mutated bands / total bands observed for loci. [b] Range of mutation rates (excludes all loci that had zero mutations). Rates are as reporte by the authors or are estimated based on the number of bands examined. [c+] Cluster mutations observed but treated as unique mutational events. [c-] Cluster mutations observed and are treated as a single mutation. [d] 27 loci were pre-screened for mutability, but only 9 were used in the final analysis.

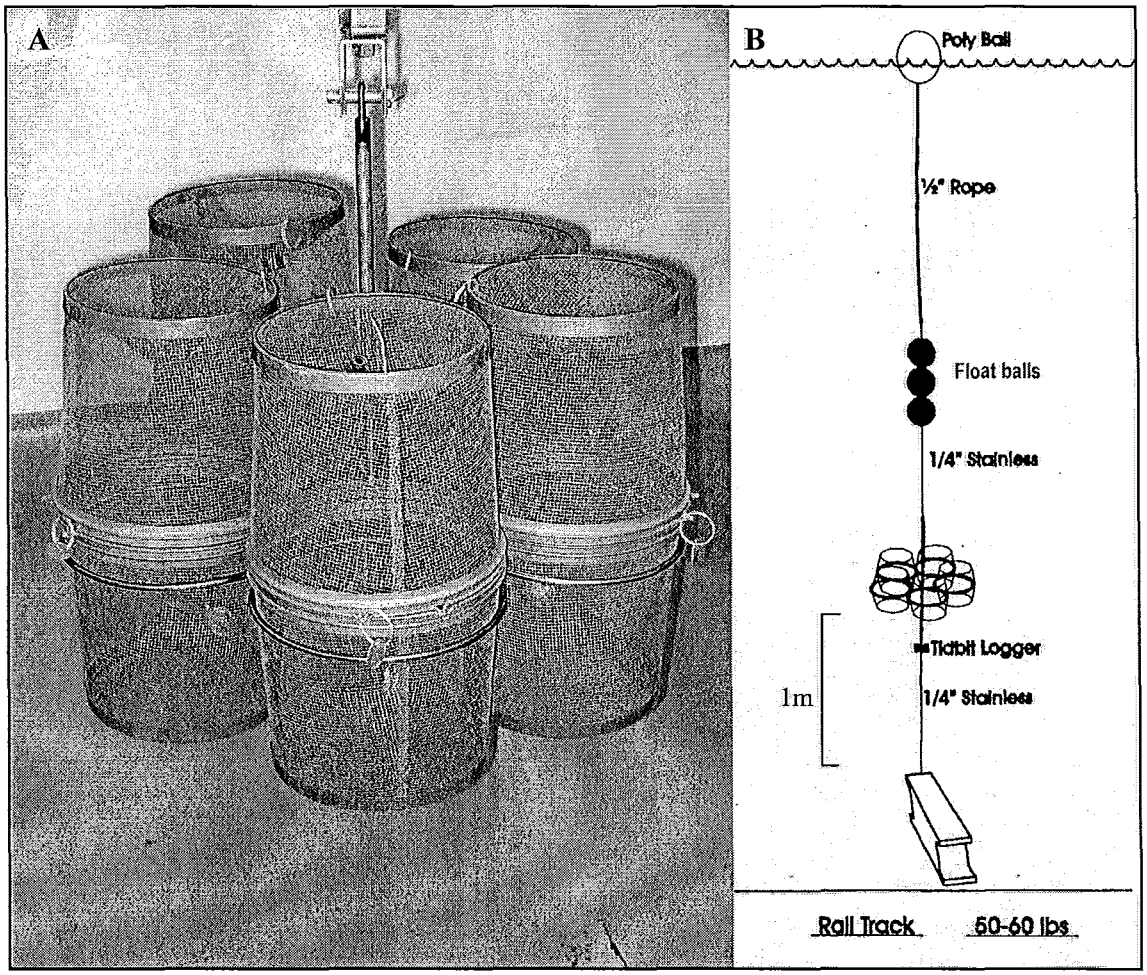


Figure 2.1: Each mooring held five modified minnow traps one meter above the sediment surface. A single mooring was used at each field site. **A**: photograph of cages positioned within a mooring. **B**: diagram of the complete mooring as used (not to scale).

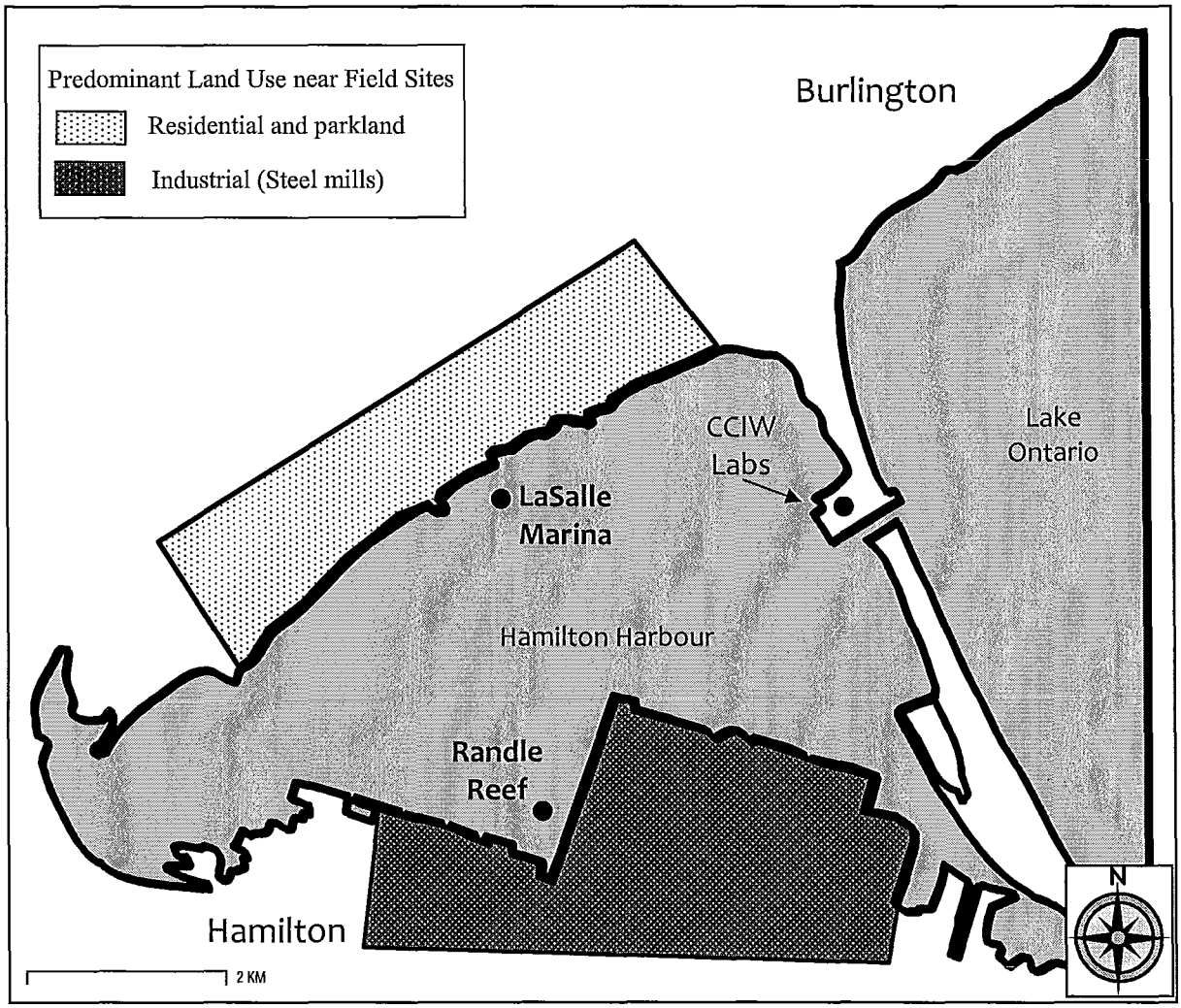


Figure 2.2: Locations of the field sites in Hamilton Harbour. Randle Reef = contaminated study site. LaSalle Marina = field control site. The fish lab was located at the Canada Centre for Inland Waters, within close proximity to both field sites.

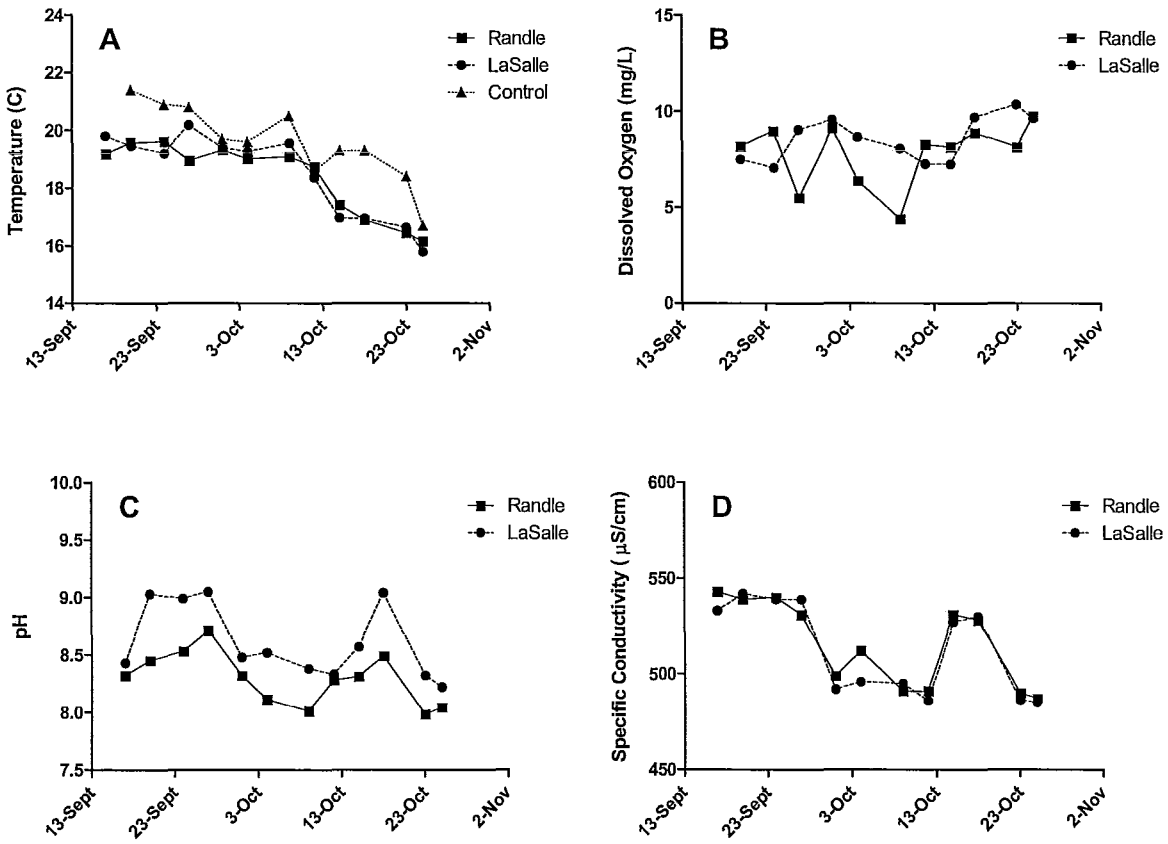


Figure 2.3: Temperature (**A**), dissolved oxygen (**B**), pH (**C**), and conductivity (**D**) profiles during the 6-week field exposure period. All field measurements were taken at cage depth (approximately 7.5 meters deep and 1 meter above sediment).

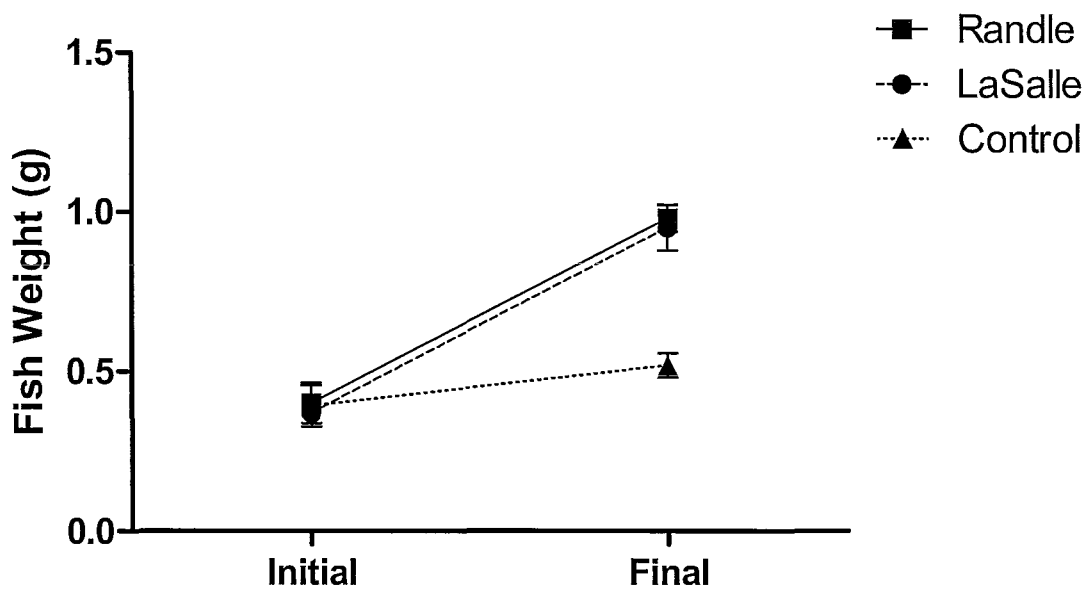


Figure 2.4: Average fish weight per treatment prior to and following the six-week field exposure period. Average fish weighs were similar in all tanks prior to exposure (ANOVA, $p = 0.531$). Average weighs increase in all groups but the increase was greater in both Randle and LaSalle replicates compared to control (Nested ANOVA, $p = 0.009$; Tukey's, $p < 0.05$). Error bars represent 95% C.I.

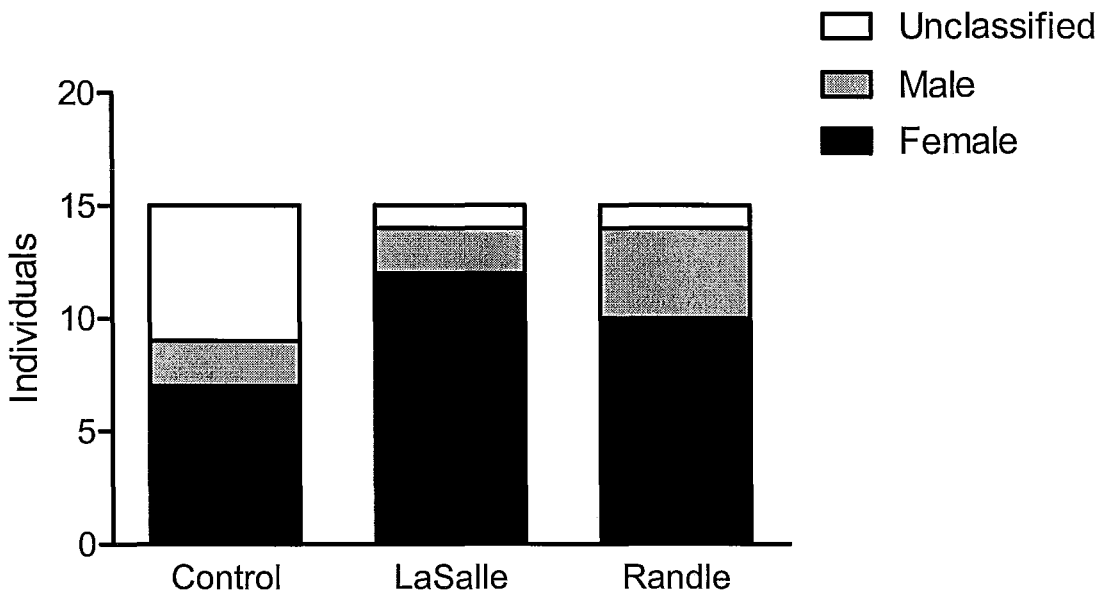


Figure 2.5: Distribution of males, females, and unclassified (immature) fish in subsets of fish dissected immediately following the *in-situ* field exposure. Sex was determined by inspections of gonads, and could not be determined for some individuals due to underdevelopment of gonads. Data shown is for all individuals dissected from each treatment (3 fish per replicate, 5 replicates per treatment). The overall distribution was not different statistically ($\chi^2 = 3.69$, $d.f. = 2$; $p = 0.073$).

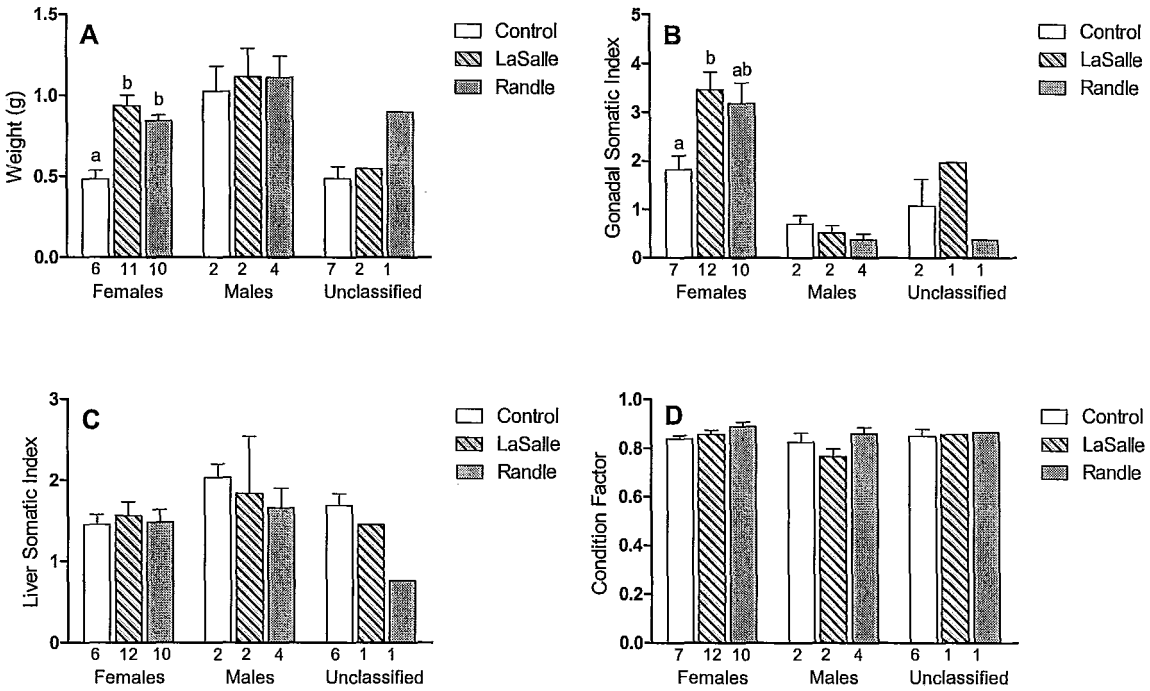


Figure 2.6: Average weight (**A**), GSI (**B**), LSI (**C**), and *K* (**D**) scores for a subset of adult fish dissected immediately following the six-week exposure period. Immature fish were excluded from the analysis but are shown for completeness. Differing letters above bars indicate groups that differed (Nested ANOVA for females or one-way ANOVA for males, $p < 0.05$, followed by Tukey's all-pairwise comparisons, $p < 0.05$). Absence of letters indicates that all values were similar amoung treatments (ANOVA $p > 0.05$). Error bars represent SEM; numbers under the x-axis indicate sample size.

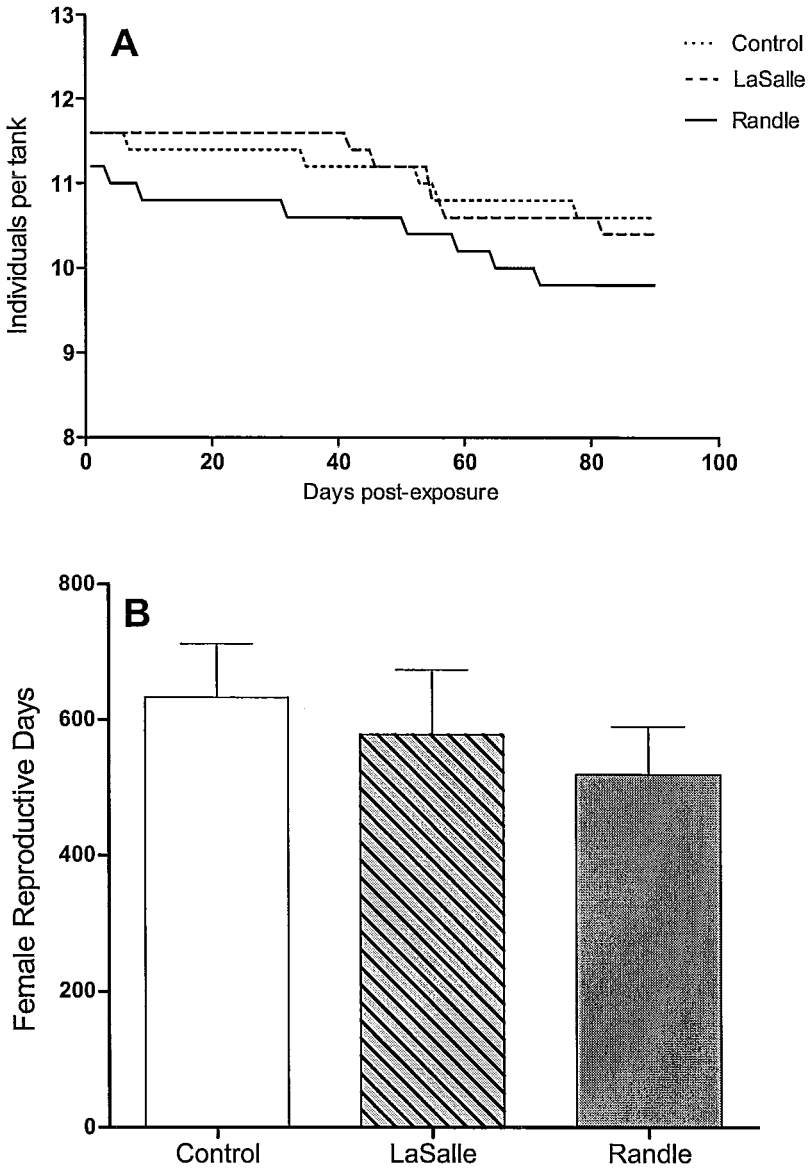


Figure 2.7A: Survival of fish in each treatment over time. Lines represent the average number of fish in each of the five replicate tanks per treatment over time. Error bars not shown for graphical clarity. **2.7B**: Average number of female reproductive days per treatment. See text for details. Errors bars represent SEM.

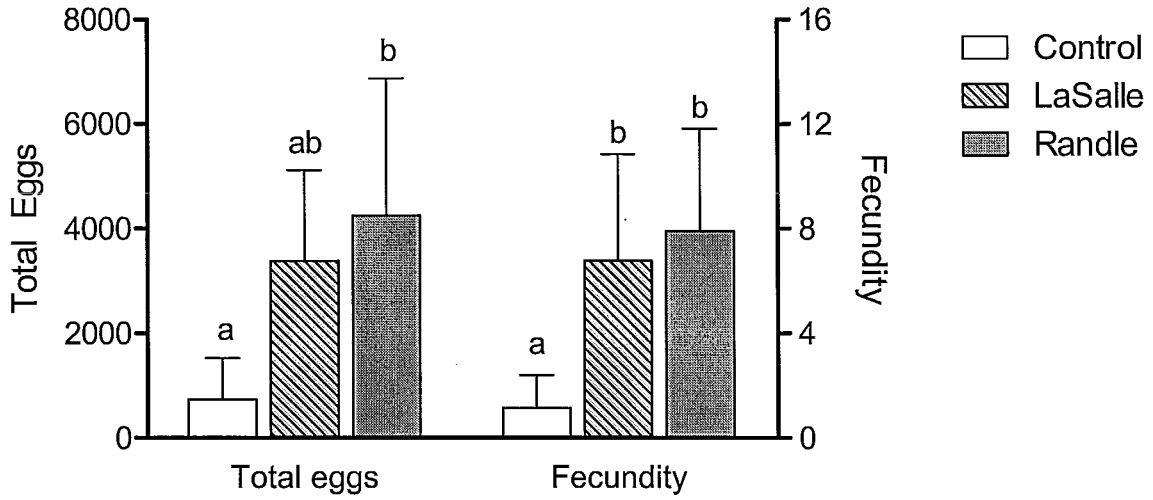


Figure 2.8: Egg production expressed as total eggs produced per replicate and as fecundity (eggs per female reproductive day). Groups differed significantly for both total eggs (ANOVA, $p = 0.029$) and fecundity (ANOVA, $p = 0.016$). Differing letters above bars indicate groups that differed when tested using Tukey's all-pairwise comparisons ($p < 0.05$). Error bars represent SEM. All treatments were statistically similar following exclusion of two Control groups that produced no eggs; see text for details.

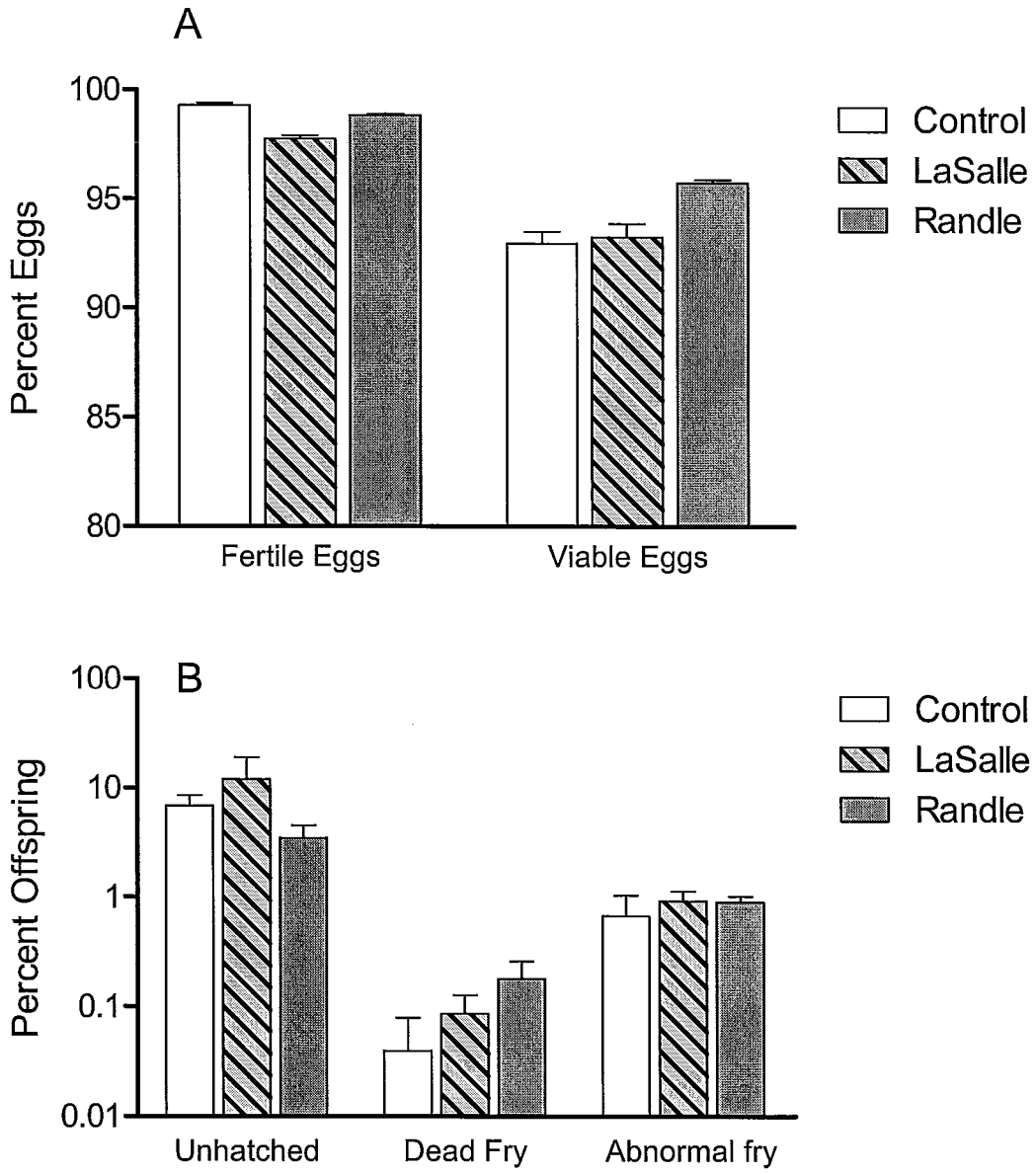


Figure 2.9A: Egg fertility and viability rates. **2.9B**: Percent of unhatched eggs, dead fry, and abnormal fry at enumeration (24-48 hours post-hatch).Error bars represent SEM. There were no statistically-significant differences in these five egg/fry quality parameters among the three treatments. (ANOVA or Kruskal-Wallis, $p > 0.05$). Error bars represent SEM.

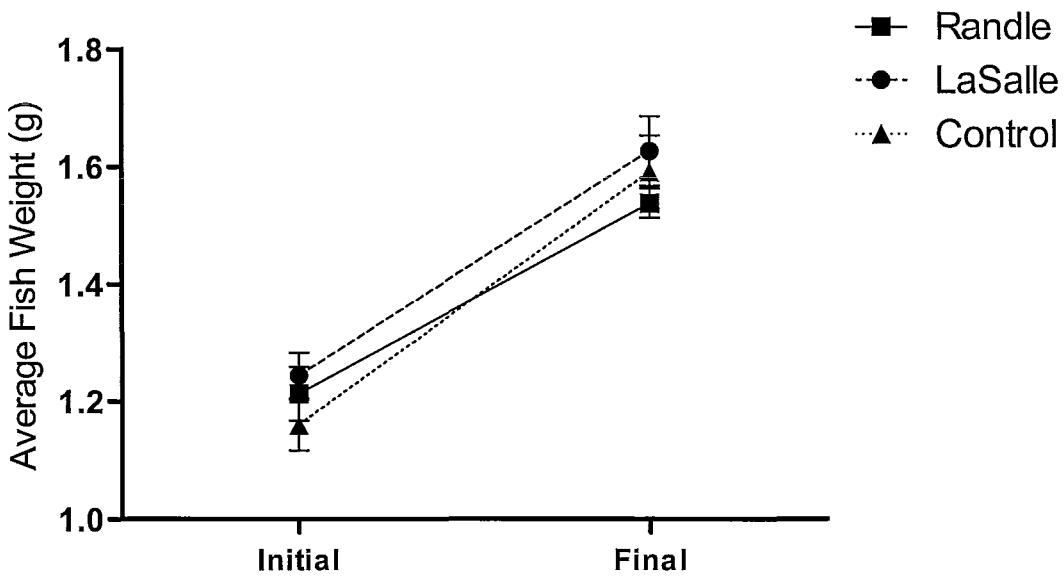


Figure 3.1: Average fish weight per treatment prior to and following the 21-day in-lab exposure period. Average fish weighs were similar in all tanks at initiation (ANOVA, $p = 0.330$) and termination of exposure (Nested ANOVA, $p = 0.648$). Error bars represent SEM. N = 4 replicates per treatment, 21-22 fish per replicate.

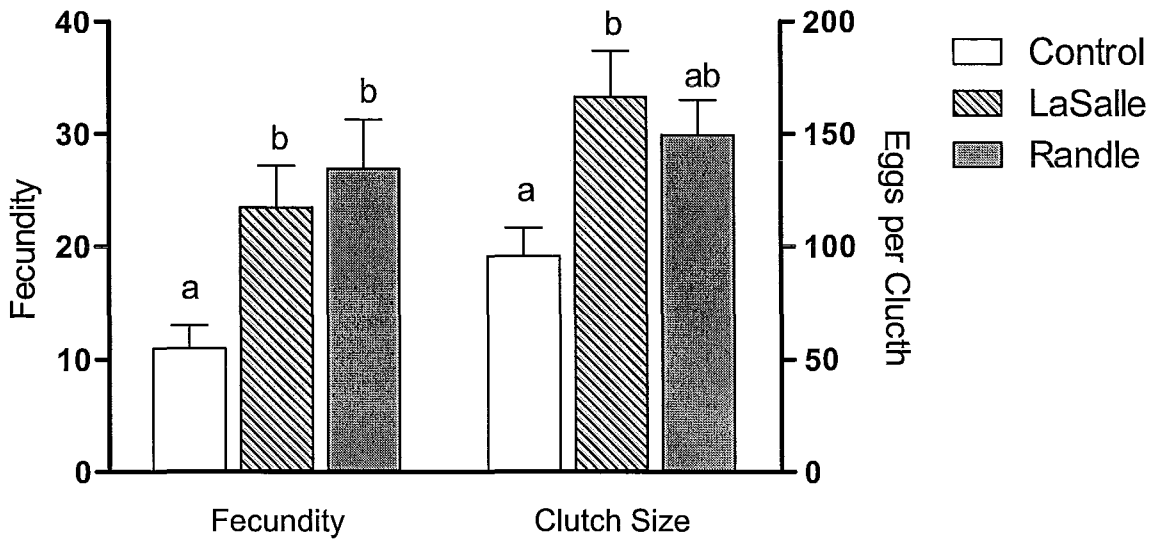


Figure 3.2: Average egg production (as fecundity: eggs per female reproductive day) and clutch sizes by treatment. Groups differed significantly for both fecundity (Nested ANOVA, $p = 0.009$) and clutch size (Nested ANOVA, $p = 0.021$). Differing letters above bars indicate groups that differed when tested using Tukey's all-pairwise comparisons ($p < 0.05$). Error bars represent SEM.

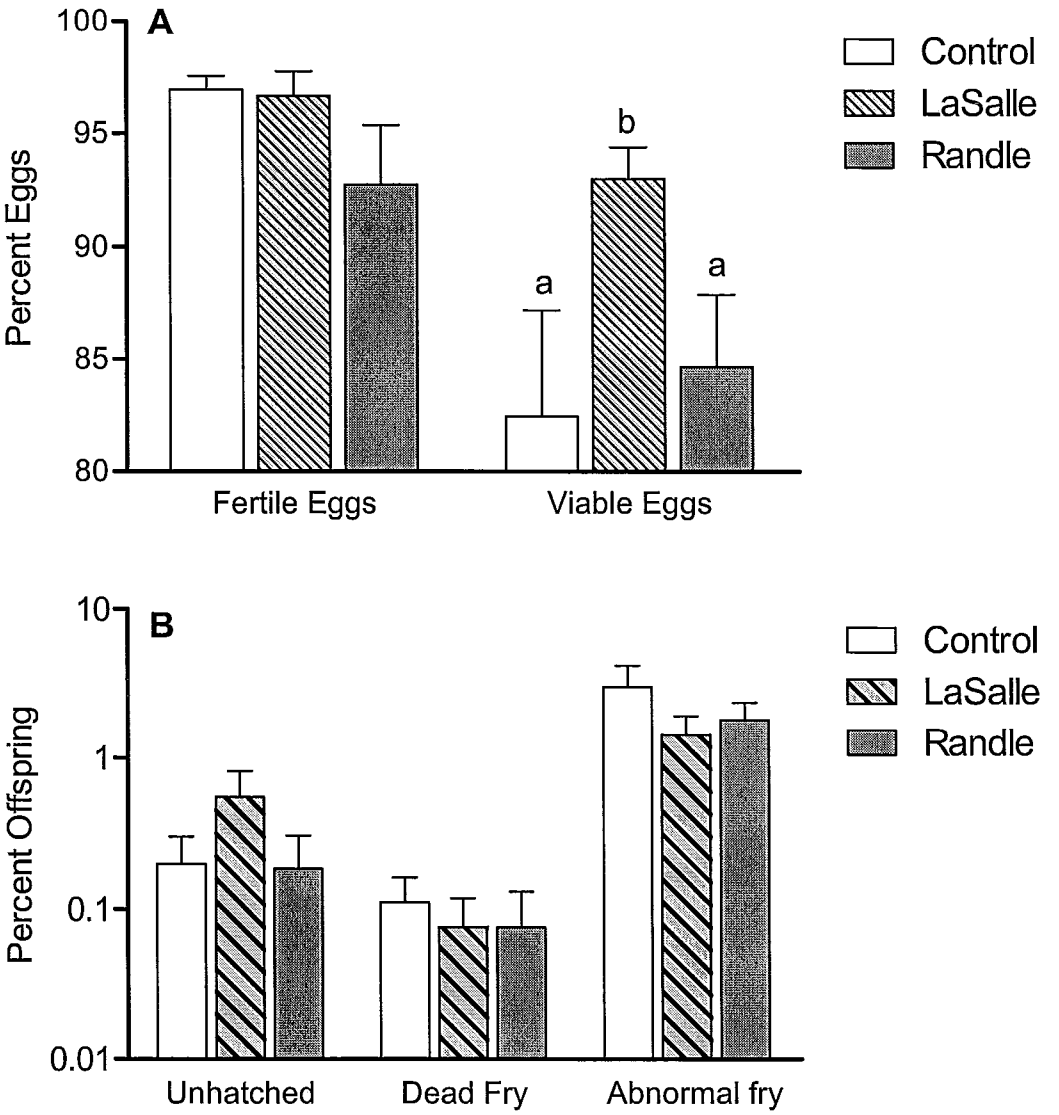


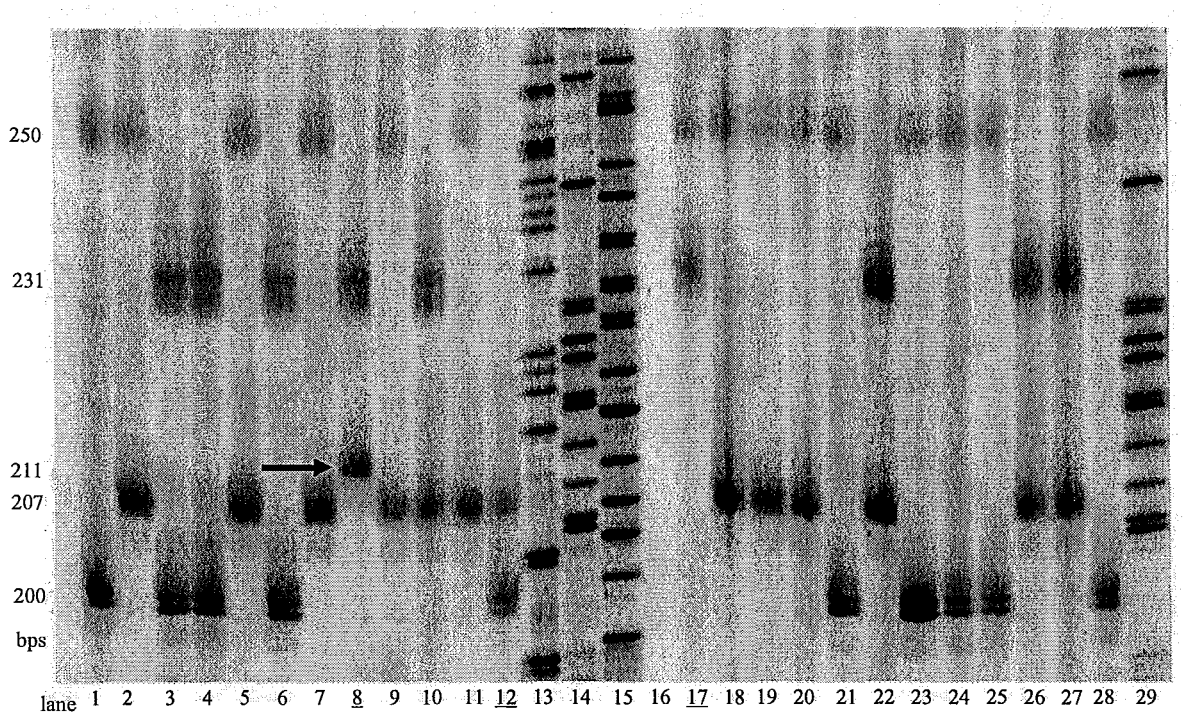
Figure 3.3A: Egg fertility and viability rates. Groups had similar fertility rates (Kruskal-Wallis, $p = 0.561$), but differed in terms of egg viability (Kruskal-Wallis, $p < 0.001$). **3.3B**: Percent of unhatched eggs, dead fry, and abnormal fry at enumeration (24-48 hours post-hatch). Error bars represent SEM. Differing letters above bars indicate groups that differed when tested using the Kruskal-Wallis all-pairwise comparisons ($p < 0.05$). Absence of letters indicates that all values were similar among treatments (Kruskal-Wallis, $p > 0.05$).

Appendix One: Glossary of Acronyms

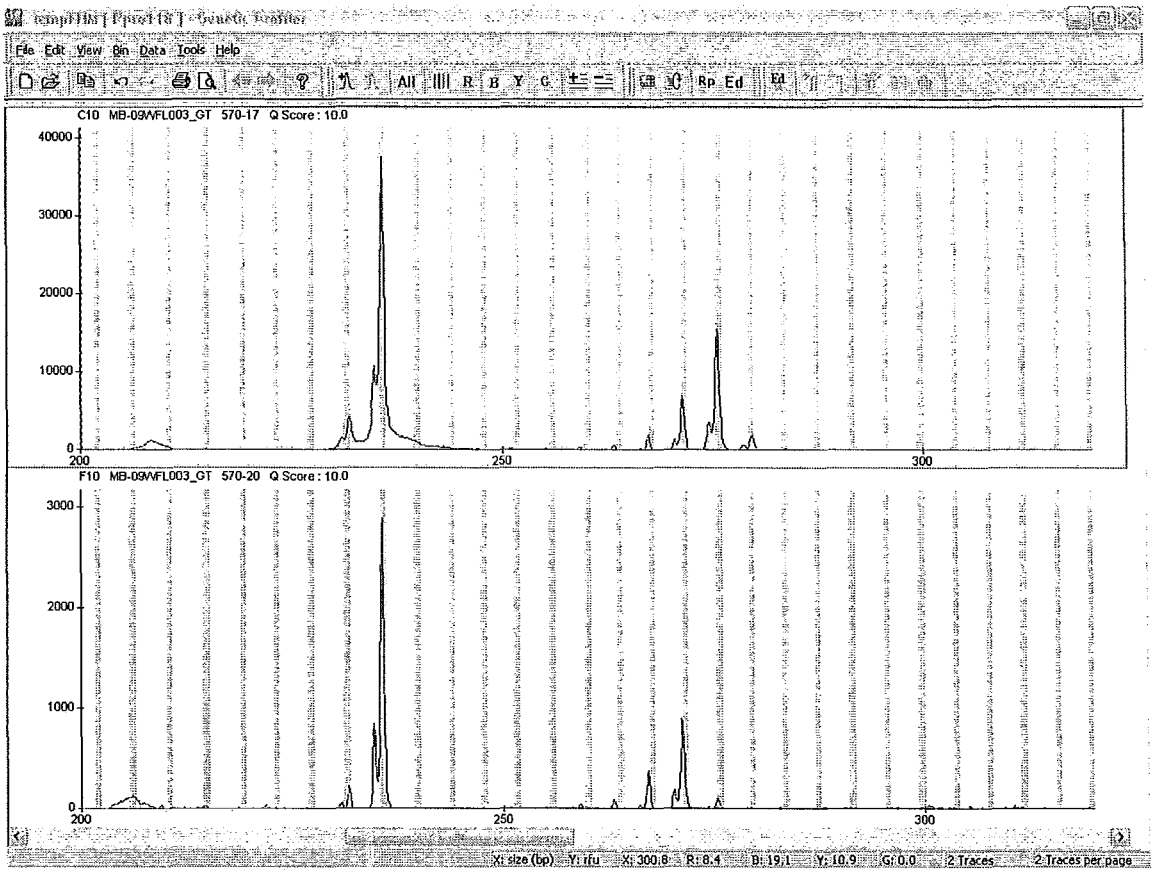
AOC	Area Of Concern. A designation given by the International Joint Commission to areas within the Great Lakes that show impairments at a number of the "Beneficial Use" criteria.
bp, bps	Base-pair, base-pairs. A measure of a DNA sequence length.
BW	Body weight.
EROD	Ethoxyresorufin-O-deethylase, a biomarker used in fish.
ESTR	Expanded-simple-tandem-repeat. A type of tandem-repeat DNA, with core repeat lengths of 2-10 bps and total lengths of 10 - 100 kbs.
FBS	Frozen brine shrimp.
GSI	Gonadal-somatic index. A measure of gonad weight relative to body weight.
hpf	Hours post-fertilization. The number of hours since a clutch of eggs was fertilized.
IJC	International Joint Commission.
K	Condition factor. A ratio of body weight to total length.
kbp	Kilobase-pairs. 1000 base-pairs.
LSI	Liver-somatic index, sometimes referred to as hepatic-somatic index. A measure of liver weight relative to body weight.
PAGE	Polyacrylamide gel electrophoresis.
PAH	Polycyclic aromatic hydrocarbons.
PCB	Polychlorinated biphenyls.
PCR	Polymerase chain reaction.
SEM	Standard error of the mean. A statistical measure of dispersal.
SL	Standard length. In this study, defined as the distance between the snout and tip of the tail.

Appendix Two:

Examples of gels showing germline mutations



Example of a PAGE gel showning a germline mutation in at *Ppro118* in fathead minnow DNA. Paternal DNA in lane 12. Maternal DNA in lane 17. Offspring DNA in lanes 1-11, 18-28. DNA ladders (pUC 18) in lanes 13-15, 29. Negative control in lane 16. A 4-bp parternally derived insertion mutation of has occurred in the individual in lane 8 (arrow).



Example of a germline mutation seen in fathead minnow DNA samples analysed using the MegaBACE capillary electrophoresis system. Shown are genetypes for two siblings with identical genotypes at the locus *Ppro118* except that the individual in the upper panel inherited a mutated allele. Genotypes: individual in upper panel: 275 / 236 (mutated allele underlined); individual in lower panel: 271 / 236. Father (not shown) 236 / 271. Mother (not shown) 227 / 236.

Appendix Three:

Review of the technique developed for caging fish in-situ

This study was unique among *in-situ* studies in that fish were exposed for a relatively extended duration (42 days) in a large natural water body. A review of the literature suggests that invertebrates are most commonly used in extended *in-situ* exposures, and most often cages are positioned in relativity shallow, wadeable waters.

However, a number of *in-situ* exposures have used fish for durations equal to or greater than 7 days in length. For example, Nichols et al. (1999) exposed fathead minnows for 21 days in small rivers, with cages placed downstream from several municipal wastewater treatment facilities. Fish were not fed during the exposure period, and only 20 – 68% of the fish were alive at the end of the exposure period. Mac et al. (1990) caged fathead minnows in the Detroit River for 10 days, and reported mortality rates of 5% (reference site) – 24% (contaminated site). In that study, cages were positioned at the sediment layer (2.4 meters depth) and halfway in the water column at each site.

Leadly et al. (1999) caged brown bullhead (*Ameiurus nebulosus*) up to 16 days in the Detroit River, without feeding, and reported elevated mortalities which prevented the study from continuing beyond day 16. Importantly, the authors attributed the elevated mortality rates to handling stress and not site contaminant, as similar mortality rates were observed at both reference and contaminated sites.

Echoles et al. (2000) maintained channel catfish (*Ictalurus punctatus*) in cages within the Saginaw River for 28 days to measure bioaccumulation of PCB's in comparison to semipermeable membrane devices, but did not report mortality rates. Conversely, Karrow et al. (2003) caged rainbow trout (*Oncorhynchus mykiss*) in Hamilton Harbour for 21 days and reported no mortalities although fish from some sites appeared lethargic. More recently, Barbee et al. (2008) caged juvenile coho salmon (*Oncorhynchus kisutch*) for seven days at a coal-tar contaminated site. No report of mortality was made, although complete loss of fish from one cage occurred during cage recovery.

Given the proposed utility of *in-situ* exposure studies (Burton et al., 2005; Crane et al., 2007), it is surprising that so few caged-fish exposures have been reported. Fish are likely avoided in favour of smaller organisms such as benthic invertebrates due to higher mortality rates in fish and increased technical requirements.

We have demonstrated an effective method for caging fish for sub-chronic durations. Fish were caged for 42 days, in relatively deep water (8.5 meters). Although the caging apparatuses were custom made, they did not require any special materials. Cages were simple minnow traps slightly modified to prevent fish from escaping. Importantly, we did not require divers to position and retrieve the cages, as was done in several other studies (Leadly et al., 1999; Echols et al., 2000; Barbee et al., 2008). This resulted in substantial saving in time and effort.

The feeding methodology used in this study was sufficient to allow fish to gain weight over the course of the 42 day exposure, and fish returned to the lab in good health.

Furthermore, fish were observed taking food while cages were still being handled. This indicates that the movement of fish and cages from the 7.5-meters depth to the surface was not overly stressful to the fish.

Finally, by using small fish and small cages, we were easily able to use true replication at each site. The five cages were arranged in close proximity and in a circular fashion, and fish in all cages at each site were most likely exposed to the same level of contaminants or other stressors. This has not been the case for most *in-situ* studies with fish, where one or two relatively large cages have been used per site.

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Appendix Four:

Utility of microsatellite in evaluating germline mutation rates

Although we failed to detect a significant increase in germline mutations, the conclusion is not necessarily that the exposure was insufficient for the elevation of germline mutation rates, but only that the exposure was insufficient to cause a detectable and significant increase in mutations within the microsatellite loci we examined. It is possible that the use of other microsatellite loci, or other heritable markers such as minisatellites and ESTR's, might have served as a more sensitive marker.

The use of microsatellites DNA markers in fish has increased dramatically in the last decade, largely driven by the needs of fisheries managers and conservation biologist (O'Connell and Wright, 1997). Unfortunately, few studies have focused on the mutation rates of these markers and fewer yet have investigated the effect of chemical or physical stressors on mutation rates.

To date, the majority of tandem repeat mutation studies have focus on humans, birds, and mice. Typically, higher mutation rates have been reported for minisatellites and ESTR's than for microsatellites. In a review of reported spontaneous germline mutation rates for microsatellites, minisatellites, and ESTR's, a generalization might be made that most microsatellite loci reveal overall lower spontaneous mutation rates compared to minisatellites and ESTR's (Table 4.1). However a few hypervariable loci have been reported with spontaneous mutation rates that approach those of the other markers (Table 4.1).

Most tandem repeat mutation studies have employed radiation as a stressor, with only a few providing evidence for chemically-induced mutations (see review by Singer et al., 2006). Not surprisingly, the chemicals used were ones already classified as mutagenic or clastogenic using other assays. For example, Vilariño-Güell et al. (2003) reported elevated ESTR mutations following single-dose injections of three potent and well established chemical mutagens, and Glen et al. (2008) demonstrated elevated ESTR mutations in mice exposed to a number of anti-cancer drugs previously shown to be clastogenic or mutagenic.

Indeed, few studies have evaluated risk for elevated germline mutations resulting from exposure to environmentally relevant chemicals. Hedenskog et al. (1997) reported elevated minisatellite germline mutations in mice exposed to PCB's, and even higher mutations rates were observed when mice were exposed to both PCB's and diesel exhaust even though diesel exhaust alone failed to induce a significant increase in mutation rates. However, tree swallows (*Tachycineta bicolor*) environmentally exposed to PCB's did not shown elevated rates of minisatellite mutations (Stapleton et al., 2001). These two studies, combined with the gull and mouse studies reported by Somers et al. (2002, 2004, 2008) and Yauk et al. (1996, 2000, 2008), appear to be the only evaluations of tandem repeat germline mutations following exposure to environmental contaminants that have been reported to date

One limitation of the method we used to detect germline mutations is that only mutations that altered allele size were detected. Point mutations, for example, would be overlooked using the current system. If point mutations such as single base substitutions

are near as common in microsatellite loci as are addition or deletion mutations, it is possible that the effects of exposure on DNA mutation rates may be underestimated. For example, Amanuma et al. (2002) reported that 54% of the observed DNA mutations at a non-repetitive transgene target in transgenic zebrafish exposed to 10 μg/ml benzo[a]pyrene were base substitutions. Similarly, transgenic medaka exposed to 0 or 120 mg/L ethylnitrosourea showed mutations within the *cII* gene that were 74% and 94% base substitutions, respectively (Winn et al., 2000). In the latter study, the authors noted that exposure to ethylnitrosourea not only lead to increased mutations (all types) but also increased the proportion of point mutations relative to insertions or deletions (Winn et al., 2000), suggesting that some mutagens may preferentially alter DNA sequences while rarely affecting the sequence length. Such changes would be missed using the current method of detection. However, these mutations occurred in non-repetitive sequences that are likely less prone to insertion and deletion mutations as are tandem repeat DNA. Furthermore, the actual rate of these mutations cannot be compared to mutation rates in microsatellites given the techniques used to isolated and identify the mutations at these transgenic loci.

Another disadvantage of microsatellites for detecting mutations is that, at least compared to the other two tandem repeat markers, a larger sample size may be needed to detect significant elevations in mutation rate. Microsatellites show mutation rates that are typically 10- to 100-times lower than what has been reported for some minisatellites and ESTR's. In a review of the utility of tandem repeat's for detecting environmental mutagens, Dubrova (reported in Wyrobek et al., 2007) estimated that a sample size of

2400 individuals would be required to detect a significant germline mutation rate increase in humans using microsatellite markers, whereas only 240 would be needed if minisatellites or ESTR's were used.

Finally, a limitation to using microsatellites, at least at this stage, is the lack of information regarding dose-response and sensitivity. By comparison, the commonly used mouse ESTR markers have been well characterized and comparisons of that system with other mutagen models such as the dominant lethal assay and the specific locus test indicate that ESTR's have a similar or more sensitive response to the several mutagens tested thus far (reviewed by Singer et al., 2006).

Microsatellite markers offer several advantages over minisatellites and ESTR markers. The single biggest advantage of microsatellites is the reduction of time, expense, and sample volume requirements that can be achieved over the other tandem repeat markers. In this study, DNA of sufficient quality and quantity was rapidly extracted from small tissue samples including 2-day old fry. Simplified extraction methods were employed which reduced the number of steps and amount of manual intervention, precluding the use of hazardous organic chemicals or specialized DNA isolation kits, and reducing the opportunities for sample cross-contamination to occur. Although not optimized to the full potential, multiple loci were often co-amplified in multiplex PCR reactions or mixed following PCR amplification, and genotyping could be automated using capillary electrophoresis and computer software applications. The ability to rapidly screen a large number of individuals at multiple loci is considered to be one of the key reasons why microsatellites are so widely employed in both human and wildlife

forensic applications (Koumi et al., 2004; Jobin et al., 2008). This is in stark contrast to minisatellites and ESTR's, which typically require large amounts of high quality DNA, although PCR techniques have been developed to amplify ESTR markers. Either way, DNA fragments or PCR products must be transferred to membranes and probed with radiolabeled makers following separation on agarose gels. Another important difference is the resolution that can be achieved using microsatellites. Exacting techniques allow for discrimination of sequences which differ by as little as 1 bp, and changes of 4 bps are easily identified. Moreover, specific allele sizes can be determined, facilitating direct comparison between loci of the same or different species.

Finally, the utility of microsatellites in forensics and population genetics has lead to a constant stream of microsatellite loci being published. For example, description and primer sequences for over 35,500 microsatellite loci were published in the journal Molecular Ecology Resources (formerly Molecular Ecology Notes) between Nov 30th 1999 and April 2nd 2009[1]. Of these, almost 2500 loci have been identified for the important fish families *Acipenser, Clupeidae, Cyprinidae, Gadidae, Pleuronectidea, and Salmonidae*. Clearly this is an underestimate of the total number of loci described to date, since these numbers only represent loci described in a single scientific journal.

In light of the advantages and limitation to using microsatellite markers versus minisatellite markers, we feel that the simplicity of use and increased resolution warrant further development and utilization of microsatellite markers for germline mutation analysis. Microsatellites have been used to show significant elevations in germline

[1] http://tomato.bio.trinity.edu/ search.php?q=microsatellite&field_name=marker_type&id=&page=1"; accessed April 8th 2009

mutation rates following accidental exposure to radiation in humans and birds (Ellegren et al., 1997; Slebos et al., 2004; da Cruz et al., 2008), as well as following experimental exposure to radiation in fish (Tsyusko et al., 2007). Characterization of more variable markers may result in increased sensitivity and reduce the number of individuals screened to a level comparable with minisatellites and ESTR's.

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